Molecular epidemiology of *Plasmodium* spp. in North Sumatera, Indonesia, and the efficacy of dihydroartemisinin-piperaquine and artemether-lumefantrine for treatment of clinical malaria

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DECLARATION

I, Inke Nadia Diniyanti Lubis, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

ABSTRACT

Intensive efforts to control malaria in the past two decades have resulted in substantially decreased morbidity and mortality in Indonesia, as in malaria endemic countries elsewhere. At present, 70% of malaria endemic regions in Indonesia have achieved low transmission status. However, stronger measures are needed to accomplish the national target of elimination by 2030. Scaling up on active surveillance using highly sensitive diagnostic tests is crucial to allow identification of low-density, asymptomatic infections, strengthened by effective treatment with artemisinin-based combination therapy (ACT) to reduce cases and halt transmission. Resistance to this drug combination, therefore, poses the greatest threat for both malaria control and the elimination goal. The aims of this research project are:

(1) to investigate the distribution of *Plasmodium* spp. in three rural areas of North Sumatera province, Indonesia, to provide information on malaria risk in the province;
(2) to identify drug resistance-associated genetic polymorphisms carried by local *Plasmodium falciparum* parasites;
(3) to evaluate the efficacy of two ACTs, dihydroartemisinin-piperaquine and artemether-lumefantrine for treatment of uncomplicated falciparum malaria.

In this study, molecular diagnostics identified four Plasmodium species (*P. vivax* 33.9%, *P. knowlesi* 32.0%, *P. falciparum* 24.8%, and *P. malariae* 9.3%) among 16.5% of participants (614/3731). *P. falciparum* populations were dominated by amodiaquine-resistant parasites, indicated by a high proportion of *pfcrt*-SVMNT and *pfmdr1* 86Y/184Y haplotypes. This study is the first to our knowledge to test for the presence of mutations in the *pfk13*-propeller domain in western Indonesia, and evidence of polymorphisms in *pfk13* was found in a small number of individuals. A Thr to Ala mutation at codon 474, was observed in six individuals, and the C580Y mutation, previously associated with reduced susceptibility to artemisinin in the Greater Mekong subregion , was seen in a single asymptomatic individual that was not part of the clinical study. Baseline marker carriage was then tested for association with treatment outcomes in the treated cohort study. Dihydroartemisinin-piperaquine and artemether-lumefantrine were both shown to be highly effective for treatment of *P. falciparum* malaria,

irrespective of the *pfk13* genotypes. Nevertheless, recurrent parasitaemia of all four species was observed in both groups at day 42, implying lack of sterilising protection from lumefantrine and piperaquine. Further studies are warranted to explore the phenotypic impact of *pfk13* mutant alleles in this region and regular monitoring of drug efficacy against all species present is required.

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CONTENTS

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

CHAPTER 7

CHAPTER 8

REFERENCES

APPENDICES

PUBLICATIONS

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

No figure.

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

CHAPTER 7

LIST OF TABLES

CHAPTER 1

CHAPTER 2

No table.

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

CHAPTER 7

ABBREVIATIONS

ß-hcg	beta human chorionic gonadotropin
µL	microliter
µM	micromolar
3D7	Wild-type *P. falciparum*
7G8	South-American mutant variant of *P. falciparum*
95% CI	95% confidence interval
ACT	Artemisinin-based combination therapy
A. balabacensis	*Anopheles balabacencis*
A. flavirostris	*Anopheles flavirostris*
A. nigerrimus	*Anopheles nigerrimus*
A. subpictus	*Anopheles subpictus*
A. sundaicus	*Anopheles sundaicus*
ACPR	Adequate clinical and parasitological response
AL	Artemether-lumefantrine
AN	Artesunate-naphthoquine
Anopheles spp.	*Anopheles* species
AQ	Amodiaquine
ARDS	Acute respiratory distress syndrome
ASAQ	Artesunate-amodiaquine
AS-MQ	Artesunate-mefloquine
AS-SP	Artesunate-sulfadoxine-pyrimethamine
bp	base pair
CRF	Case record form
Cytb	*Cytochrome b*
CQ	Chloroquine
Dd2	African/Southeast Asian mutant variant of *P. falciparum*
DHA	Dihydroartemisinin
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotides

DP	Dihydroartemisinin-piperaquine
G6PD	Glucose-6-phosphate dehydrogenase
GCP	Good clinical pracite
Glurp	Glutamate-rich protein
GMS	Greater Mekong sub-region
g	gram
GWAS	Genome-wide association study
Hb	Haemoglobin
HumTuBB	Human ß-tubulin gene
IC_{50}	50% inhibitory concentration
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
IPTi	Intermittent preventive treatment in infants
IPTp	Intermittent preventive treatment in pregnancy
IRS	Indoor residual spraying
ITN	Insecticide-treated net
K13	Kelch 13
kg	kilogram
LAMP	Loop mediated isothermal amplification
LSHTM	London School of Hygiene and Tropical Medicine
MDA	Mass drug administration
mg	milligram
mITT	Modified intention to treat
MQ	Mefloquine
Msp1	Merozoite surface protein 1
Msp2	Merozoite surface protein 2
ng	nanogram
nM	nanomolar
OR	Odds ratio
P. coatneyi	*Plasmodium coatneyi*
P. cynomolgi	*Plasmodium cynomolgi*
P. falciparum	*Plasmodium falciparum*
P. fieldi	*Plasmodium fieldi*
P. inui	*Plasmodium inui*

P. knowlesi	*Plasmodium knowlesi*
P. malariae	*Plasmodium malariae*
P. ovale curtisi	*Plasmodium ovale curtisi*
P. ovale wallikeri	*Plasmodium ovale wallikeri*
P. vivax	*Plasmodium vivax*
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PCT	Parasite clearance time
Pfatp6	Sarco/endoplasmic reticulum Ca^{2+} - ATPase orthologue of *P. falciparum*
*Pf*API	*Plasmodium falciparum* annual parasite incidence
Pfap2-mu	*Plasmodium falciparum mu* chain of the AP2 adaptor protein complex gene
Pfcrt	*Plasmodium falciparum* chloroquine resistance transporter
Pfdhfr	*Plasmodium falciparum* dihydrofolate reductase gene
Pfdhps	*Plasmodium falciparum* dihydropteroate synthase gene
Pfk13	*Plasmodium falciparum* kelch 13 gene
Pfmdr1	*Plasmodium falciparum* multidrug resistance protein 1 gene
Pfmrp1	*Plasmodium falciparum* multidrug resistance-associated 1 gene
PfPR_{2-10}	*Plasmodium falciparum* parasite rate in 2-10 years old
Pftctp	*Plasmodium falciparum* translationally controlled tumour protein
Pfubp1	*Plasmodium falciparum* ubiquitin carboxyl terminal hydrolase
Pgh1	P-glycoprotein homologue 1
PgMET	*Plasmodium* genus methionine tRNA gene sequence
Plasmodium spp.	*Plasmodium* species
pLDH	Parasite lactate dehydrogenase
*Pv*API	*Plasmodium vivax* annual parasite incidence
PvPR_{1-99}	*Plasmodium vivax* parasite rate in 1-99 years old
PRR_{50}	Parasite reduction rate to 50%
PRR_{90}	Parasite reduction rate to 90%
PSA	Piperaquine survival assay
QN	Quinine
qPCR	Quantitative polymerase chain reaction
RSA_{0-3h}	Ring-stage survival assay

RDTs	Rapid diagnostic tests
RR	Risk ratio
rRNA	Ribosomal ribonucleic acid
SD	Standard deviation
SERCaP	Single exposure radical cure and prophylaxis
Sicavar	Schizont-infected cell agglutination variant antigents
SNPs	Single-nucleotie polymorphisms
SP	Sulfadoxine-pyrimethamine
TBE	Tris/borate/EDTA
WBC	White blood cell
WHO	World Health Organization
ZFN	Zinc-finger nuclease

LIST OF PUBLICATIONS

Isaac Ghinai, Jackie Cook, Teddy Tun Win Hla, Hein Myat Thu Htet, Tom Hall, **Inke Nadia Diniyanti Lubis**, Rosanna Ghinai, Therese Hesketh, Ye Naung, Mya Mya Lwin, Tint Swe Latt, David L. Heymann, Colin J. Sutherland, Chris Drakeley, Nigel Field. *Malaria epidemiology in central Myanmar: identification of a multi-species asymptomatic reservoir of infection*. Malaria Journal. 2017;16:16-25.

Inke Nadia Diniyanti Lubis, Hendri Wijaya, Munar Lubis, Chairuddin P. Lubis, Khalid B. Beshir, Colin J. Sutherland. *Contribution of Plasmodium knowlesi to Multispecies Human Malaria Infections in North Sumatera, Indonesia.* The Journal of Infectious Diseases. 2017;215(7):1148-1155.

Chapter 1

INTRODUCTION

1 INTRODUCTION

1.1 Malaria Worldwide

Malaria, a preventable and treatable condition, remains as the most important parasitic disease globally. In 2016, it was still endemic in 91 countries, and placing 3.8 billion people at risk. Significant progress has been made due to aggressive malaria control and elimination efforts since 2000 resulting in global reduction of 41% in morbidity and 62% in mortality. Nevertheless, the World Health Organization estimates there were still 212 million cases in 2015 with 429,000 deaths owing to this disease, most of which occurred in Africa. Seventy-one percent of all malaria deaths were in children under 5 years of age. Thus malaria continues to be viewed as a highly significant disease of a global public health importance.[1]

1.1.1 Geographical Distribution

Malaria is a vector-borne disease relying on particular conditions determined by ecological factors including temperature, rainfall and humidity for the survival of the parasites and vectors.[2] There are six major *Plasmodium* species infecting human; *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale curtisi, Plasmodium ovale wallikeri* and *Plasmodium knowlesi*[3]; transmitted by 70 of 465 known anopheles species.[4] *P. falciparum* and *P. vivax* are the two main causes of human malaria infections[5], with the former species accounting for the majority of total malaria cases especially in Africa (Figure 1.1.A).[6-8] Falciparum malaria poses a risk of severe complications and contributes to the majority of deaths.[5] *P. vivax* has the widest geographical range of all malaria parasite species, being found throughout the temperate climate zones (Figure 1.1.B).[2, 9-11] However to cause infection, this species relies on Duffy antigen to invade host reticulocytes, therefore Duffy-negative individuals, which are highly frequent in parts of Africa, are protected from *P. vivax* infection.[12, 13] *P. vivax* contributed 4% of total global cases in 2015, but outside Africa the proportion is 41% among all malaria infections.[1] Its high burden of disease is maintained in

part due to dormant liver stage parasite forms known as hypnozoites which can induce clinical relapse episodes.[14]

P. malariae and *P. ovale* spp. are less common malaria species infecting humans. These species are more prevalent in the African region, although these species are also regularly reported in Southeast Asia and the Western Pacific.[15, 16] The development of species-specific polymerase chain reaction (PCR) has helped to discriminate human malaria species and has enabled more understanding of their global distribution and prevalence. Furthermore, it has been used to identify the dimorphic forms of the sympatric *P. ovale curtisi* and *P. ovale wallikeri*.[3, 15] Both *P. malariae* and *P. ovale* spp. occur in multi-species endemic settings with a prevalence of less than 1% in most settings, but it also has been reported to be as high as 22% for malariae.[16, 17]

P. knowlesi is a malaria parasite transmitted by *Anopheles leucosphyrus* from long-tailed and pig-tailed macaques to humans.[18] The natural hosts and vectors of *P. knowlesi* are widespread throughout Southeast Asia (Figure 1.2.).[18-20] Epidemiological studies to define the distribution extent of this species in human are still limited and mainly from Malaysian Borneo, but small numbers of cases has also been confirmed by molecular diagnostic tests in the rest of the Southeast Asia region.[20-30]

Small numbers of cases of other *Plasmodium* parasites infecting humans have also been reported either experimentally or naturally by *Plasmodium cynomolgi*[31-34], *Plasmodium inui*[35], *Plasmodium brasilianum*[36], *Plasmodium simium*[37, 38], and *Plasmodium schwetzi*[39].

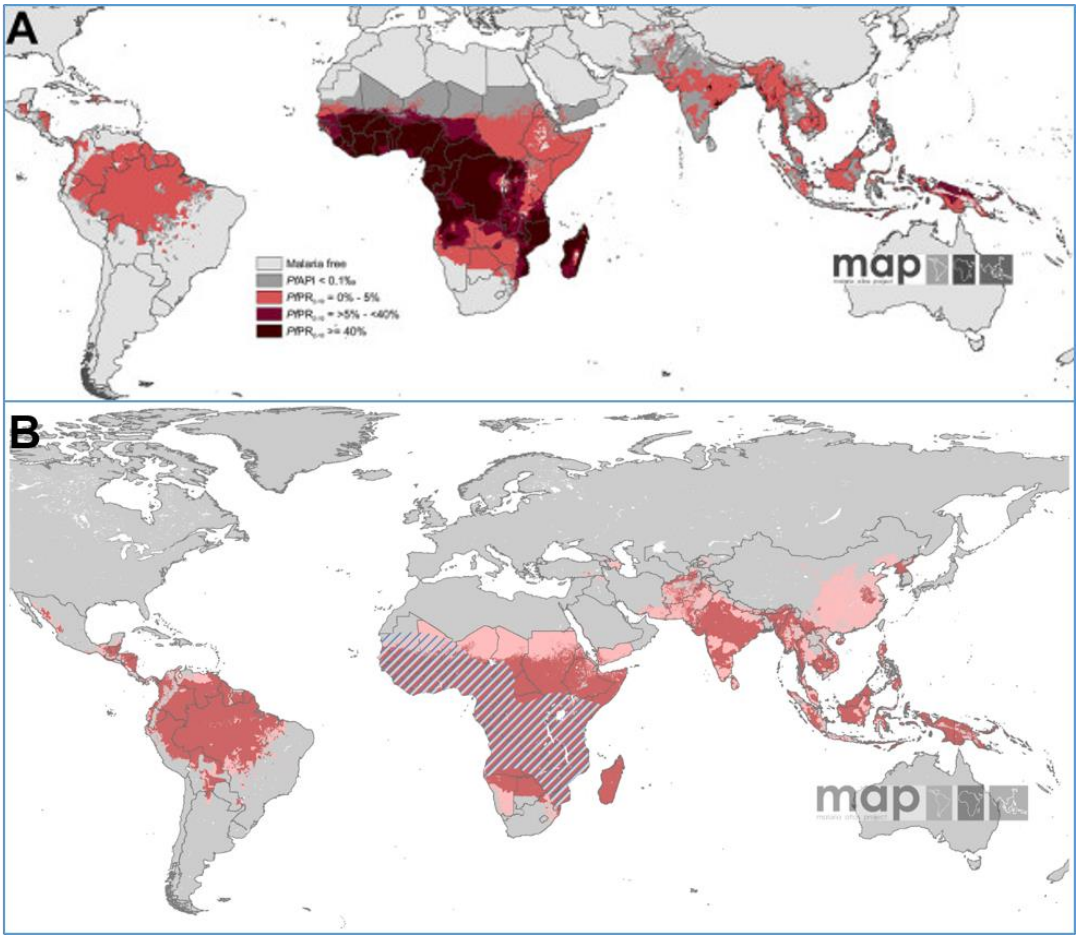


Figure 1.1. The spatial distribution of *P. falciparum* and *P. vivax* malaria. (A) *P. falciparum* malaria risk categorized as low risk (light red, where *P. falciparum* parasite rate in 2-10 year olds, *Pf*PR2-10 ≤ 5%), intermediate risk (medium red, where *Pf*PR2-10 > 5% to <40%), and high risk (dark red, where *Pf*PR2-10 ≥ 40%). The rest of the land area was defined as unstable risk (medium grey areas, where *P. falciparum* annual parasite incidence, *Pf*API <0.1 per 1,000 p.a.) or no risk (light grey); (B) *P. vivax* malaria risk defined by *P. vivax* annual parasite incidence, *Pv*API data; transmission was defined as stable (red areas, where *Pv*API ≥ 0.1 per 1,000 people p.a.), unstable (pink areas, where *Pv*API < 0.1 per 1,000 p.a.) or no risk (grey areas). Predicted Duffy negativity layers are overlaid on the *P. vivax* limits of transmission, areas where Duffy negativity prevalence was estimated as ≥ 90.% are hatched. Adapted from reference [2, 7].

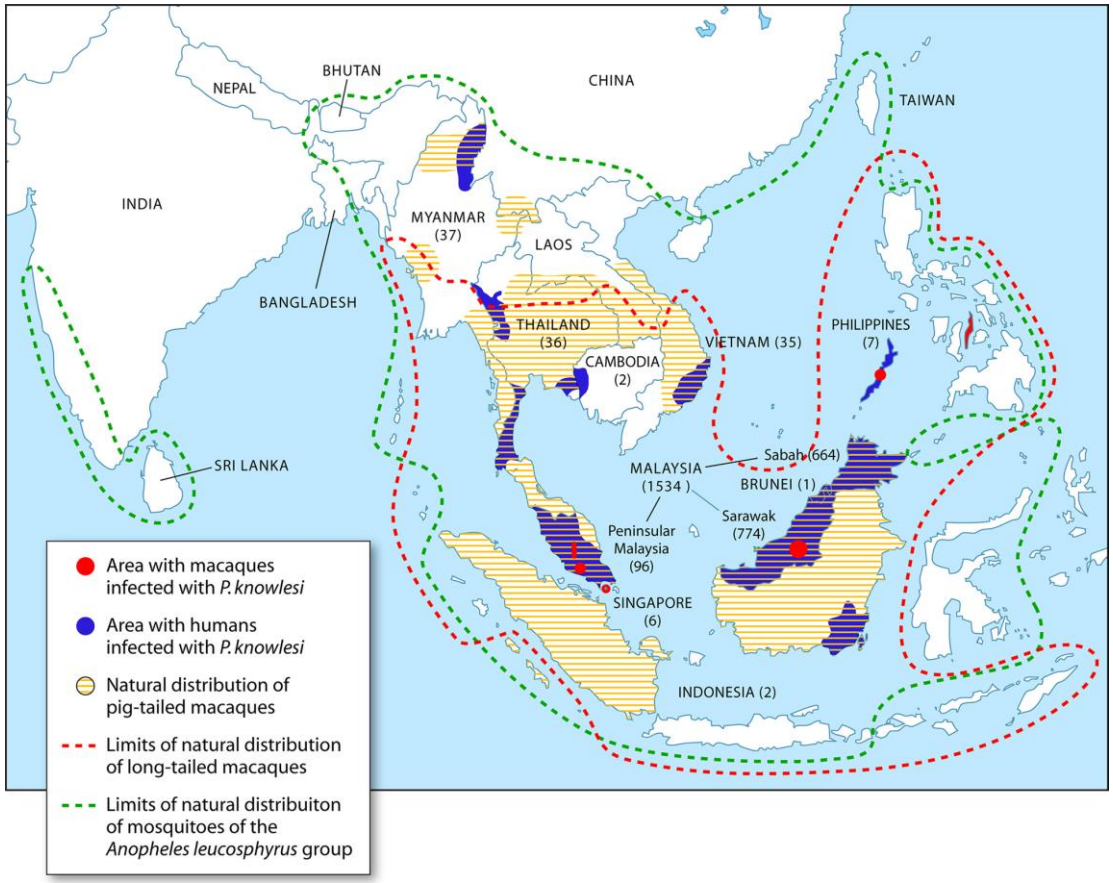


Figure 1.2. *P. knowlesi* infections reported in humans and macaques and the limits of natural distribution of two species of macaques and mosquitoe vectors of the Anopheles leucosphyrus group. Adapted from reference [40].

1.1.2 Changing Epidemiology

In the year 2000, a global commitment was made to fight malaria involving increased coverage of insecticide-treated nets (ITNs), indoor residual spraying (IRS) and prompt treatment of clinical malaria with effective antimalarial drugs, such as artemisinin-based combination therapy (ACT).[41] Since then dramatic changes in malaria risk have been seen, resulting in a remarkable decrease in prevalence and incidence. *P. falciparum* as the cause of vast majority of infections had a decline in parasite rate in 2 to 10 year olds (*Pf*PR_{2-10}) from 33% in 2000 to 16% in 2015 in the African region. Moreover, 26 of 43 mainland endemic countries in the continent reached > 50% reduction in incidence, resulting in a change in transmission status to hypoendemic with *Pf*PR_{2-10} of 1-10%, as reflected in a six-fold increase in the number of countries with low risk compared to in 2000.[42, 43] A decline in malaria transmission also corresponds to a significant decrease in the prevalence of asymptomatic infections in infants and children. A study of 760 participants aged between 2 weeks and 99 years over a 20-year period (1990-2010) in a high transmission area in Senegal showed the proportion of asymptomatic infants and children slide-positive for malaria reduced dramatically from 70% and 100% in 1990, respectively, to 1% at the end of the study.[44] However, in low endemic areas, asymptomatic submicroscopic infections also have been shown to be important through the use of molecular diagnostics.[45]

The true epidemiology of *P. vivax* is more difficult to determine. First, many of *P. vivax* cases are asymptomatic with low parasite densities which lead to underdiagnosis.[9] The hypnozoite forms of *P. vivax*, which delay the release of merozoites into the blood stream to establish detectable erythrocytic infection, also activate after different dormant periods. Those with a long latency period allows them to survive the temperate weather, and hence sustain the transmission.[46] This characteristic makes them more difficult to control using the same known measures used to control *P. falciparum*. Furthermore, it is known that low frequency Duffy-positive individuals in the majority Duffy-negative African population are sufficient to maintain transmission. Interestingly, recent evidence revealed some *P. vivax* infections among Duffy-negative individuals, historically thought to be resistant.[47-49] Given a modified estimation of only 50% rather than 100% protection by Duffy negativity from acquiring *P. vivax*, the number of population estimated to be at risk of *P. vivax* increased from 2,484,800 to 2,868,900.[50]

P. malariae and *P. ovale* spp. have much lower endemicity than the former two species, however despite the control measures in the last few years these species are not yet eliminated. There is a gap of knowledge on how these parasites maintain circulation.[15] *P. malariae* causes chronic infection that can last for years and the slower life cycle at 72-hour rate gives the advantage to avoid drug pressure from antimalarials with shorter half-lives. *In vivo* studies have shown *P. malariae* parasites survived the 3-day course treatment after being exposed with the current recommended ACT.[51, 52] *P. ovale* spp., on the other hand, have the advantage of having the dormant stage hypnozoites although there is a lack of evidence on the dormancy periods of both species before relapses occur.[53] Similar to *P. vivax*, this unique characteristic plays a role in sustaining transmission, as well as hiding the true burden of the disease.[15, 16]

In the light of elimination efforts in Southeast Asia, molecular assays have increasingly been used to identify low-level infections in surveillance studies. In this region, successful malaria control has dramatically reduced both *P. falciparum* and *P. vivax* cases.[54, 55] The numbers of *P. malariae* cases were increasingly detected by microscopy in Malaysian Borneo, and initially thought to be the common malaria infection replacing the former two.[18] Nevertheless, species-specific PCR assay confirmed the presence of *P. knowlesi* infections among these malariae cases including cases retrieved from 1996, implying that *P. knowlesi* in human is not a "new" emerging disease.[56, 57] Increased human risk to the surviving, more efficient vectors following a combination of deforestation and change in land use might have resulted in the apparent rise of confirmed human cases.[58, 59] As neighbouring countries are now moving towards elimination phase with increased use of molecular tests, a similar pattern with a significant proportion of *P. knowlesi* cases among malaria infected populations has been observed.[22, 28-30]

1.2 Life Cycle of *Plasmodium* spp.

Human *Plasmodium* spp. share a complex life cycle involving asexual and sexual developmental stages in their natural hosts: female *Anopheles* mosquito vector and human host, with macaque hosts limited to *P. knowlesi*. The transmission cycle among all *Plasmodium* spp. is similar, except the hypnozoite form that is specific in *P. vivax* and *P. ovale* spp. It starts when the female mosquitoes transmit parasites by inoculating sporozoites from their salivary glands into the human bloodstream. Motile sporozoites travel to the liver and invade

hepatocytes where a single schizont can produce 10,000 to 30,000 merozoites. This stage is referred to as the pre-erythrocytic stage. After about a week, the hepatic schizonts spp. rupture and merozoites infect the erythrocytes. This asexual cycle in the erythrocytes differs among malaria species. The cycle is 48 hours for *P. falciparum*, *P. vivax* and *P. ovale* spp., 72 hours for *P. malariae*, and 24 hours for *P. knowlesi,* respectively. When infected erythrocytes rupture, the release of merozoites results in repeated invasion of other erythrocytes.[5, 60] This phase is responsible for the clinical manifestations of malaria. Erythrocytes infected with *P. falciparum* have the special ability to sequester by adhesion to the endothelium causing microvasculature flow disruption, which often manifests as cerebral malaria, but also can cause problems in other organs especially the spleen, liver, kidney and placenta.[61-63] Some merozoites, following erythrocyte invasion, develop into sexual forms called gametocytes that can be transmitted back into the mosquito and continue their life cycle. In the mosquito gut, these gametocytes release male and female gametes that undergo fertilization and form a zygote. An ookinete later develops from the transient zygote, and then traverses the gut wall to become an oocyst. The oocyst ruptures and produces sporozoites which migrate to the salivary glands and are inoculated into potential vertebrate hosts during subsequent blood-feeding. This entire cycle from the inoculation of sporozoites to formation of new sporozoites takes a minimum of one month (Figure 1.3.).[5, 60] For *P. vivax* and *P. ovale* spp., the latent periods of the hypnozoites vary. *P. vivax* relapses depend on the geographical origin, in tropical areas it may relapse within 3-4 weeks, but may last longer up to 8-10 months in parasite strains adapted to temperate weather.[5, 46]

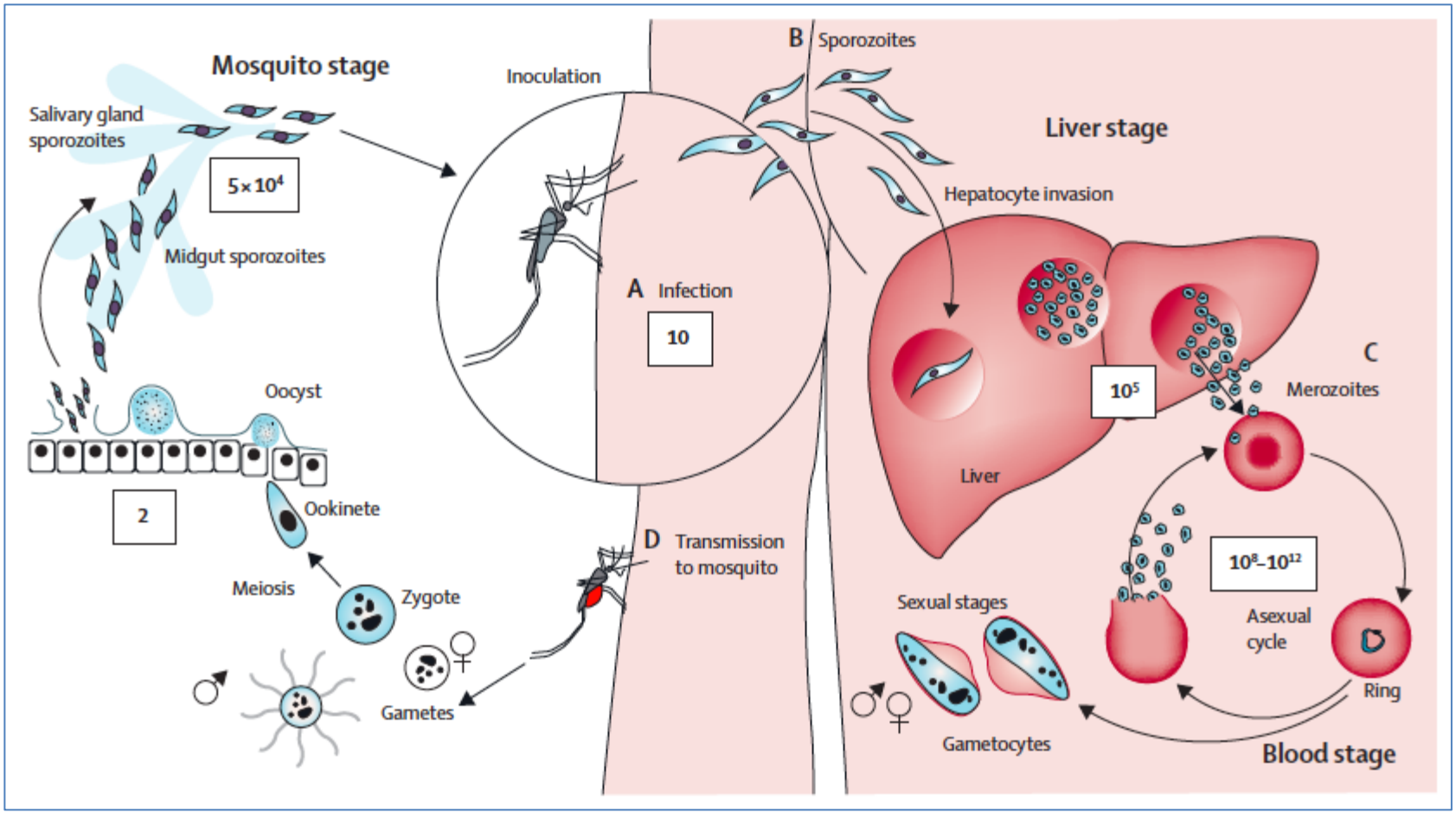


Figure 1.3. Life cycle of *P. falciparum*. Adapted from to reference [5].

1.3 Clinical Features

The first symptoms of malaria are nonspecific and characterized by headache, fatigue, abdominal discomfort, and muscle and joint aches, followed by fever, chills, perspiration, anorexia, vomiting and worsening malaise. These features often lead to overdiagnosis of malaria in developing countries, where diagnosis is frequently based only on clinical judgement with limited resources for parasitological testing.[64, 65] In children, conversely, these manifestations of uncomplicated malaria can be misinterpreted and attributed to other prevalent infections like pneumonia, gastroenteritis, and also sepsis.[66] Anaemia is also a common manifestation particularly in infants with *P. vivax* and in children with *P. knowlesi* infection.[67-69]

Severe malaria is not exclusively a disease of children, but also occurs in non-immune people across all age groups.[70] In low unstable transmission areas in Asia, severe malaria develops in all age groups due to lack of protective immunity. Features such as hyperparasitaemia, jaundice and renal insufficiency were the most common manifestations associated with increasing age.[71] In Tanzanian children, a higher density of parasites was found in severe cases but was not always linked to severe malaria. They are at the highest risk of developing severe malaria in the first 6 months of age and most severe cases present with respiratory distress, convulsion or severe anemia.[72] Coma and acidosis present equally in both older and younger ages, and are predictors for mortality.[71]

Although all *Plasmodium* infection other than *P. falciparum* were thought to be benign, some studies have revealed that severe forms of malaria exhibited as cerebral malaria, acute respiratory distress syndrome (ARDS), liver dysfunction and renal failure occurred in *P. vivax* cases and included some fatal cases.[73-75] More recently, some mortality has also been reported in *P. knowlesi* cases[76], with severe cases associated with hyperparasitaemia, jaundice, respiratory distress, hypotension and acute kidney injury similar to those features in severe falciparum malaria. However, different to severe vivax and falciparum, severe and complicated knowlesi is more likely to present in adults. The mechanisms of these parasites to cause severe disease are still not well understood. However, the previous reputation of both *P. vivax a*nd *P. knowlesi* to cause only uncomplicated manifestations are now dismissed, as they have shown their ability to develop to severe diseases.[77, 78]

1.3.1 Asymptomatic and submicroscopic infections

In high transmission areas, high and repeated exposure to parasites have an impact on the acquisition of immunity resulting in a high proportion of asymptomatic infections particularly in older children and adults.[79] With the shift of intensity due to effective malaria control in many countries, the prevalence of patent parasitaemia has decreased. Molecular techniques have been increasingly used to detect parasites and revealed a high burden of submicroscopic infections in low-endemic settings.[45, 80] The extent of these low-density infections still needs to be investigated, but they have been associated with anaemia, and premature birth and low birth weight in pregnant women and infants.[81-84] These reservoirs, more importantly, have shown to be infectious and contribute to sustaining transmission particularly in low and very low transmission settings.[80, 85]

1.4 *Plasmodium* spp. endemicity in Indonesia

The focus of this research is *P. falciparum*, therefore from this section onwards discussion will be extensively for this species. Indonesia is an archipelago that lies at the southeastern tip of Asia between West Malaysia and Papua New Guinea. It has 17,504 islands with a total land area of 1.9 million km^2.[86] It comprises 34 provinces with 416 regencies/districts and 98 cities across the country.[87] The Wallace Line, named after its discoverer, straddles between Kalimantan (Indonesian Borneo) and Sulawasi separating the western part of Indonesia from the eastern part, which have distinct biogeographical features.[88]

The climate in Indonesia is equatorial, which is typically characterised by high temperature and relatively high humidity, with heavy rainfall from October to March. This climate favours survival of *Anopheles* spp. mosquitoes and allows malaria parasites to develop in the mosquito more rapidly.[89, 90] There are 20 anopheline species documented as malaria vectors in Indonesia with overlapping distribution in all the main islands (Figure 1.4.). *Anopheles balabacensis*, *A. flavirostris*, *A. nigerrimus*, *A. subpictus* and *A. sundaicus* are the vectors found circulating in both western and eastern Indonesia.[91]

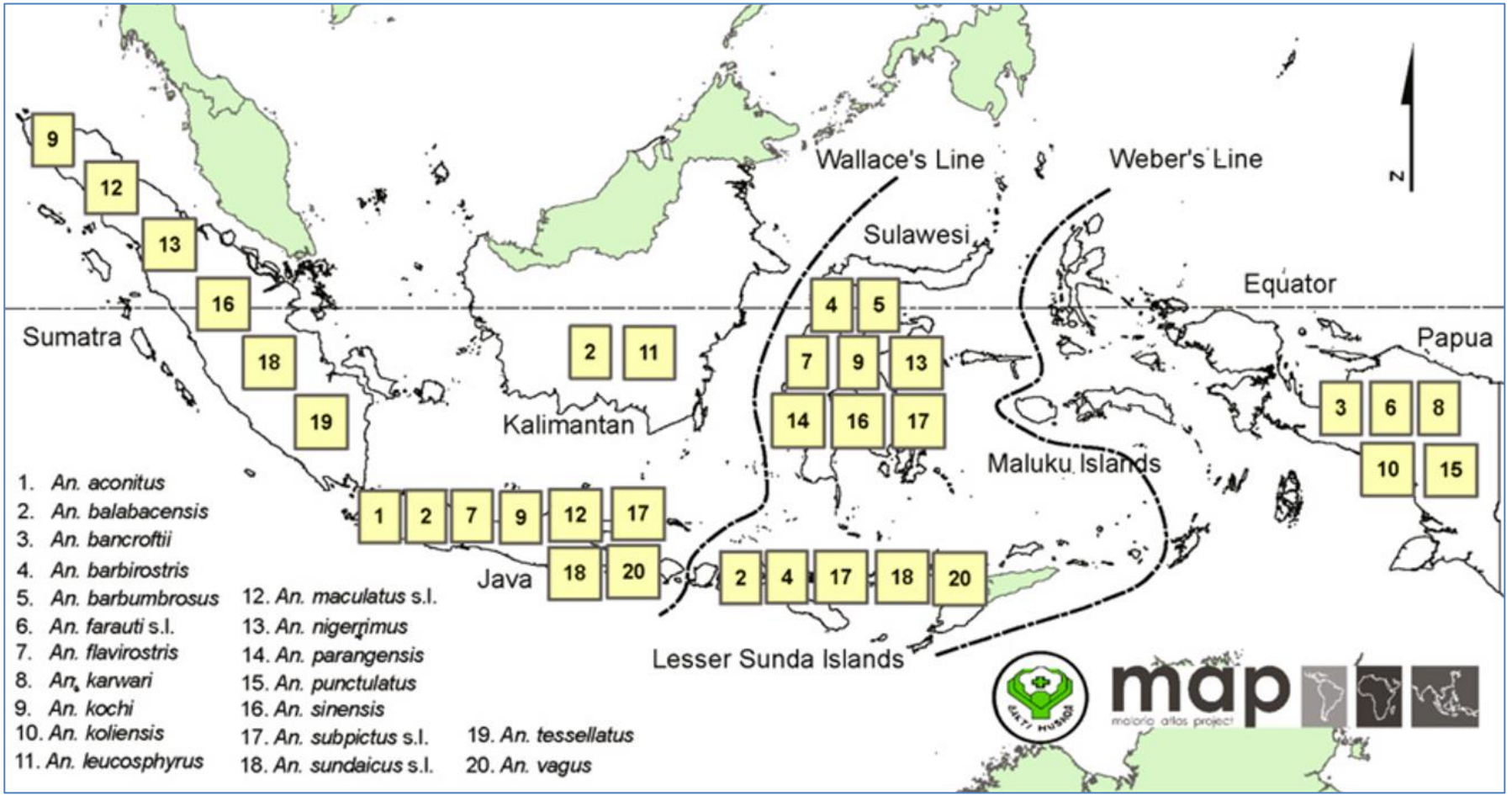


Figure 1.4. Map of the distribution of primary *Anopheles* malaria vectors in Indonesia. Adapted from reference [91].

In Southeast Asia, Indonesia contributed 9% of all malaria cases, and has the second highest burden of disease after India.[92] WHO estimated that 27% of the 257,563,815 Indonesian population live in malaria endemic areas.[1] This proportion is much lower than the estimation made by the national malaria control programmes that 85% of the population are at any risk to acquire malaria. This estimation, however, was performed without proper stratification and therefore potentially overestimated the proportion to be at risk.[92] There are difficulties in estimating the real burden of malaria in Indonesia due to changes in diagnostic testing and reporting that have occurred over time. In 2015, the country estimated 1.3 million malaria cases, a decline from 4 million cases estimated in 2000.[1, 93] The scale up of community coverage for malaria diagnosis had contributed to the increased trends of cases and deaths until 2010, while the decline of malaria cases in the following years was a result of the intensified interventions (Figure 1.5.).[92]

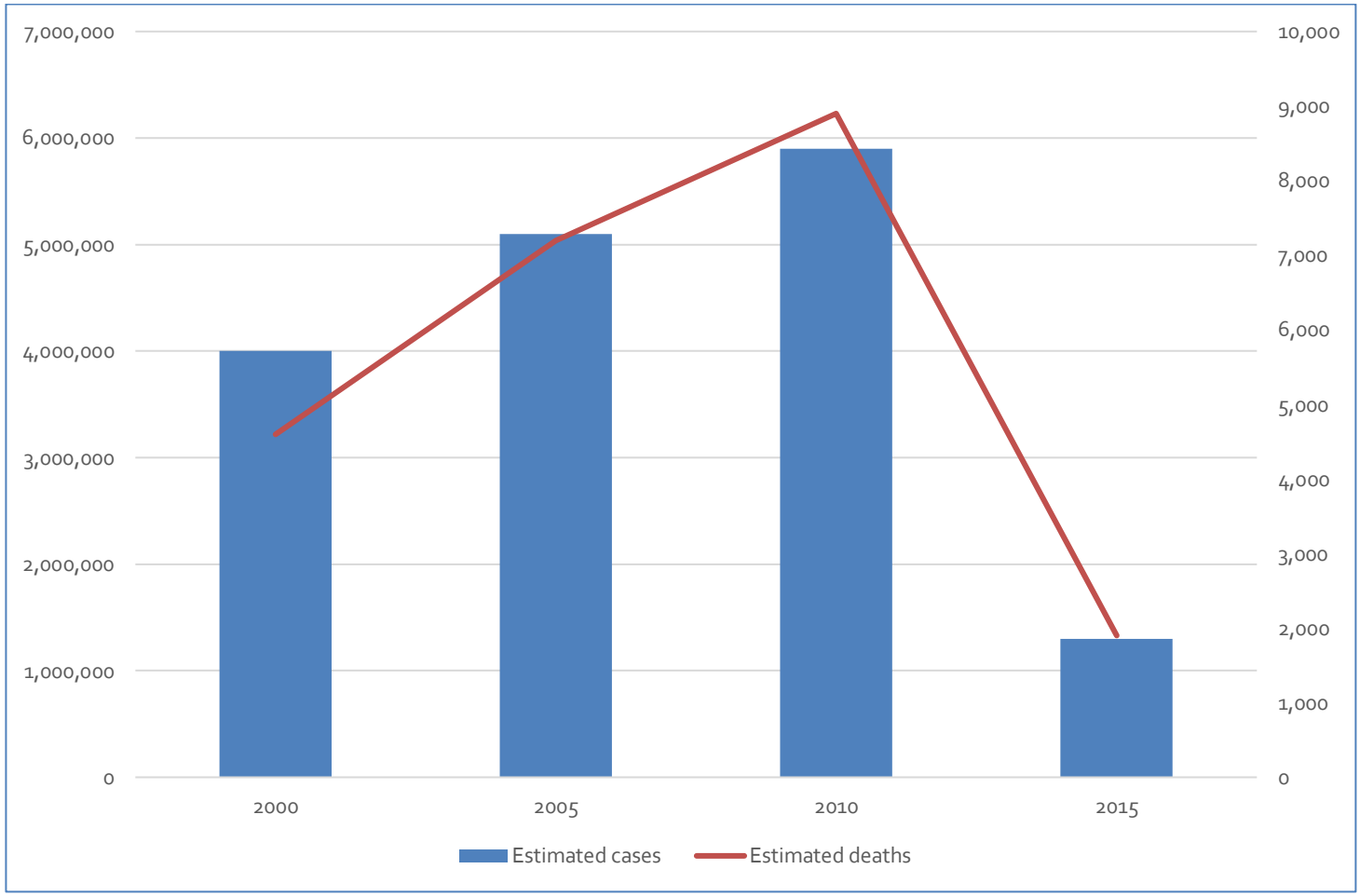


Figure 1.5. Annual malaria incidence in Indonesia, 2000-2010. Based on data from reference [1].

All species of *Plasmodium* have been documented in Indonesia, including small numbers of human *P. knowlesi* cases in Kalimantan and Sumatera islands.[21, 86] *P. falciparum* is slightly more prevalent than *P. vivax* with proportions of each 55% and 44%, respectively. However, in most regions except Papua, *P. vivax* is the predominant species. *P. malariae* and *P. ovale* spp. are rarely documented, and almost all cases were recorded in eastern Indonesia.[86, 92, 94-96]

Most of the endemic regions in the country are stable transmission zones with low transmission risk.[1] However, in eastern Indonesia the malaria transmission is more concentrated than the rest of the country, and is thus the location of most malaria surveys.[86] The total areas at risk for *P. falciparum* (87.4% vs 90.5%) and *P. vivax* (87.6% vs 93.3%) are similar between western and eastern Indonesia (Figure 1.6.).[88, 97] However, the prevalence of infection varies.[94, 98-110] The mean prevalence of *P. falciparum* was higher in eastern Indonesia at 8.14% compared to 3.06% in the western region.[88] And the mean *P. vivax* parasite rate in 1-99 year olds ($PvPR_{1-99}$) in eastern parts was at 5.6% compared to 1.5% in the western.[97] Nevertheless, the highly density of populations in western Indonesia attributes more people to be at risk of the disease (Figure 1.7.).[88]

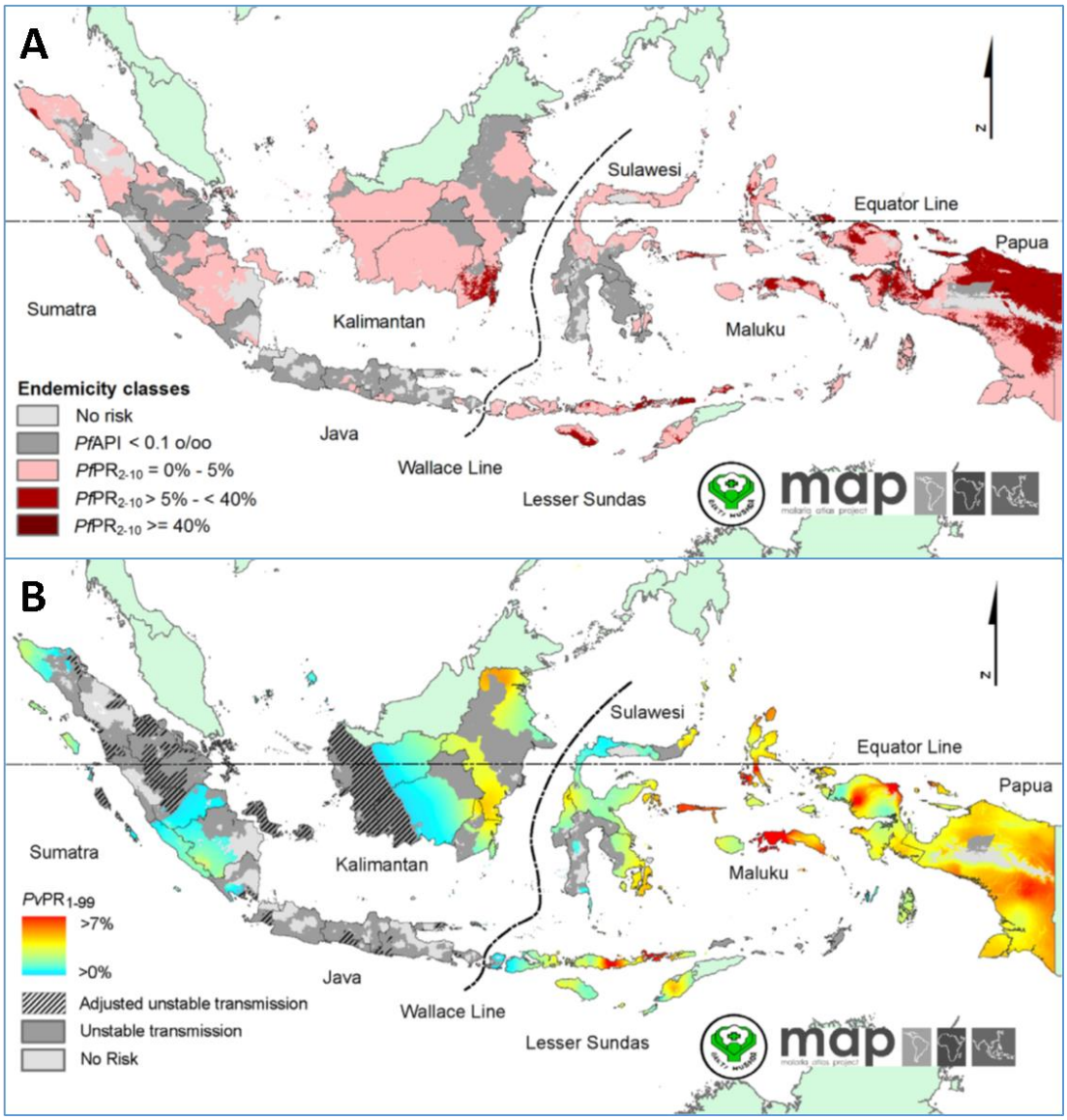


Figure 1.6. The risk map of *P. falciparum* (A) and *P. vivax* (B) malaria in Indonesia. Adapted from reference [88, 97].

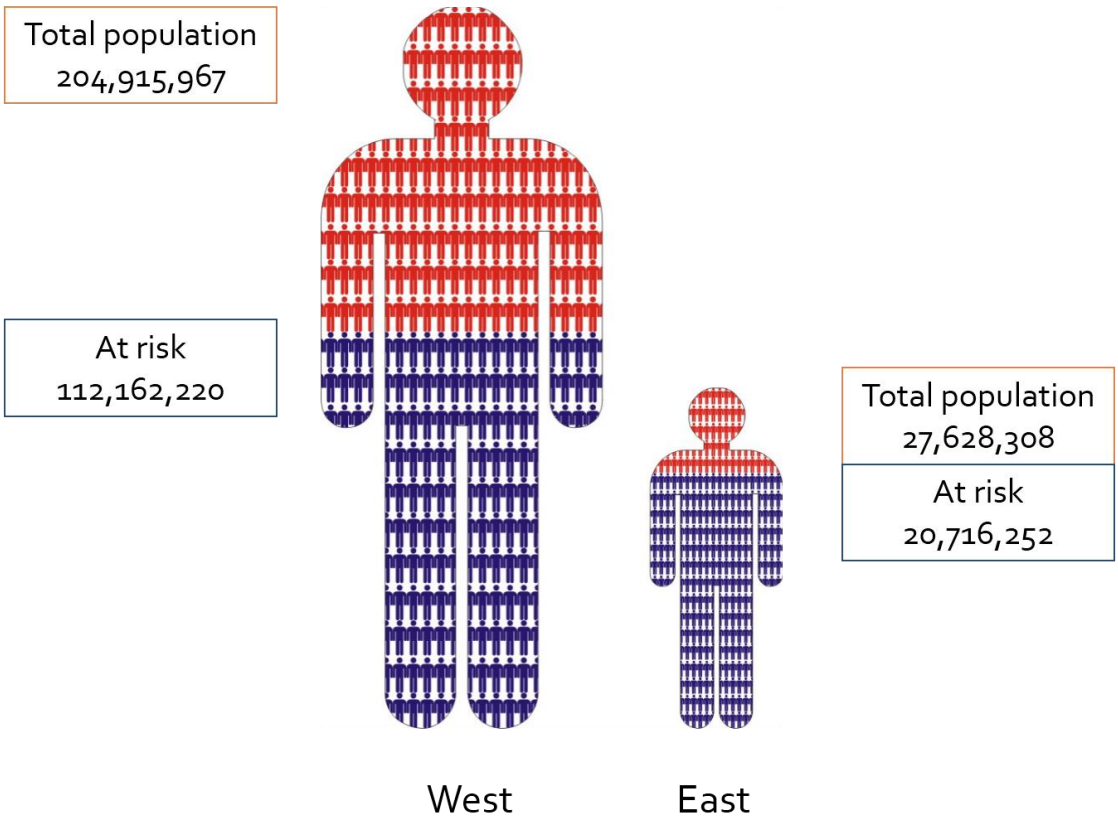


Figure 1.7. Population at risk for *P. falciparum* malaria infection in west and east Indonesia (based on data from reference [88]).

1.5 Malaria elimination and control strategies

1.5.1 Malaria surveillance system in Indonesia

Health care services in Indonesia comprise primary health centres, public hospitals, and private sector healthcare facilities. The malaria cases reporting system relies almost entirely on passive case detection. Primary health centres are located at sub-district level, with support from health posts (a facility run by a nurse) and village delivery posts (a facility run by a midwife) to increase community coverage. At primary health centres, subjects with suspected malaria infection are examined by microscopy or rapid diagnostic tests (RDTs) for confirmed diagnosis. The primary health centres generate monthly malaria reports to be sent to the district malaria control officer. The district health office then compiles the data and sends the report to the provincial health department and to the Sub-Directorate of Malaria Control at the Ministry of Health. Additional malaria data are also collected from laboratories in hospitals.[86, 92]

1.5.2 Elimination and control programmes

Malaria control programmes in Indonesia have operated since 1945, however the shift to an elimination plan was not started until 2009. The national malaria control programmes targets malaria elimination across the country by 2030, and outlined the goal into 4 milestones (Figure 1.8.).[86, 92] Hay *et al* developed a recommendation for malaria control strategies to guide countries to interrupt malaria transmission and to help transition from malaria control to elimination (Figure 1.9.).[111] In Indonesia where the level of malaria transmission varies, interventions have to be adapted to the local contexts.[88] The government's plans on elimination strategies includes the efforts to equip all primary health centres with the capacity for malaria diagnosis and treatment by 2010, implementation of active and passive case detections with periodic mass surveys, case management with effective drugs, vector control, and improvement in surveillance system.[86]

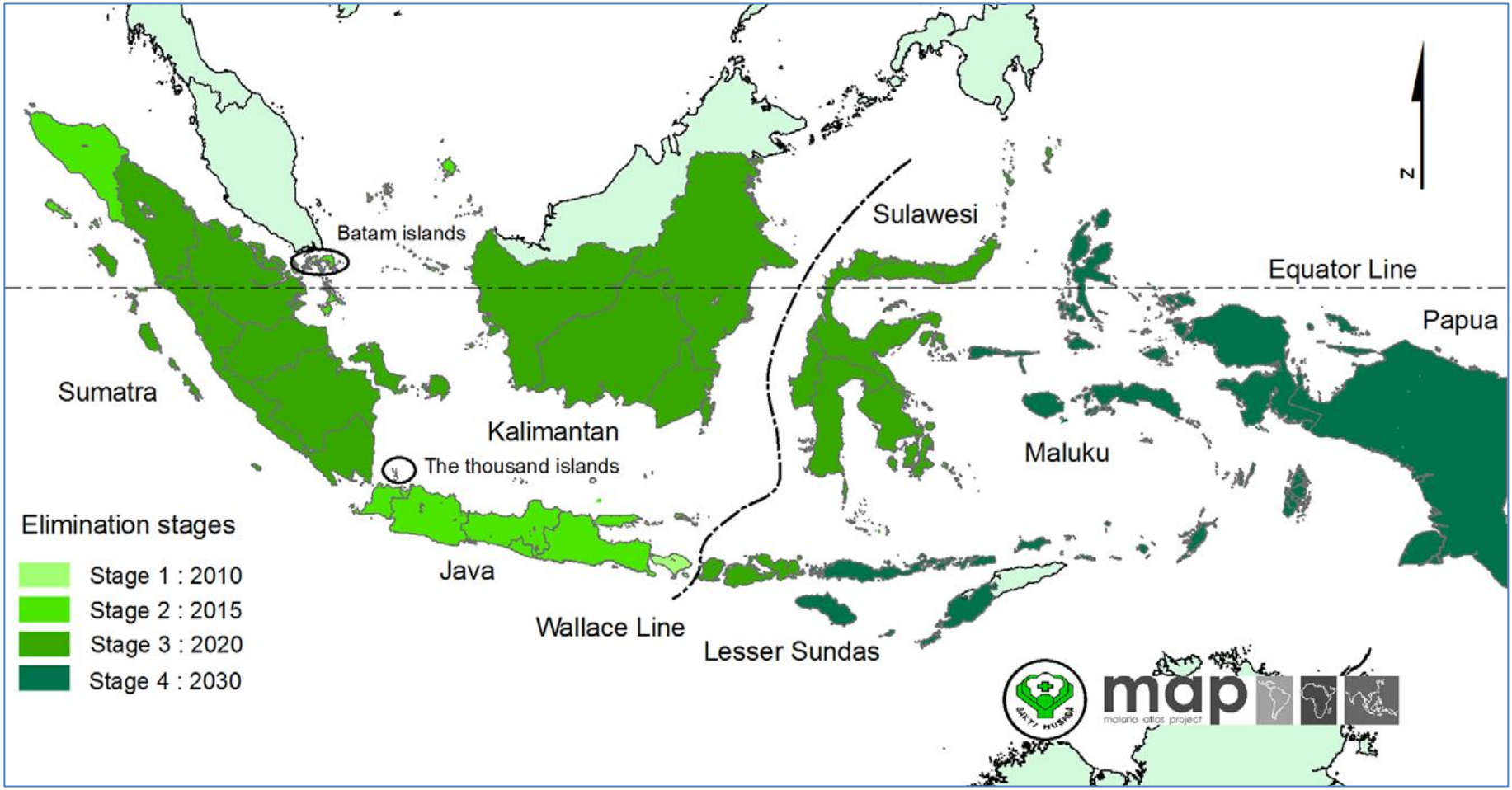


Figure 1.8. The map of Indonesian provincial border with the elimination objectives. Adapted from reference [88].

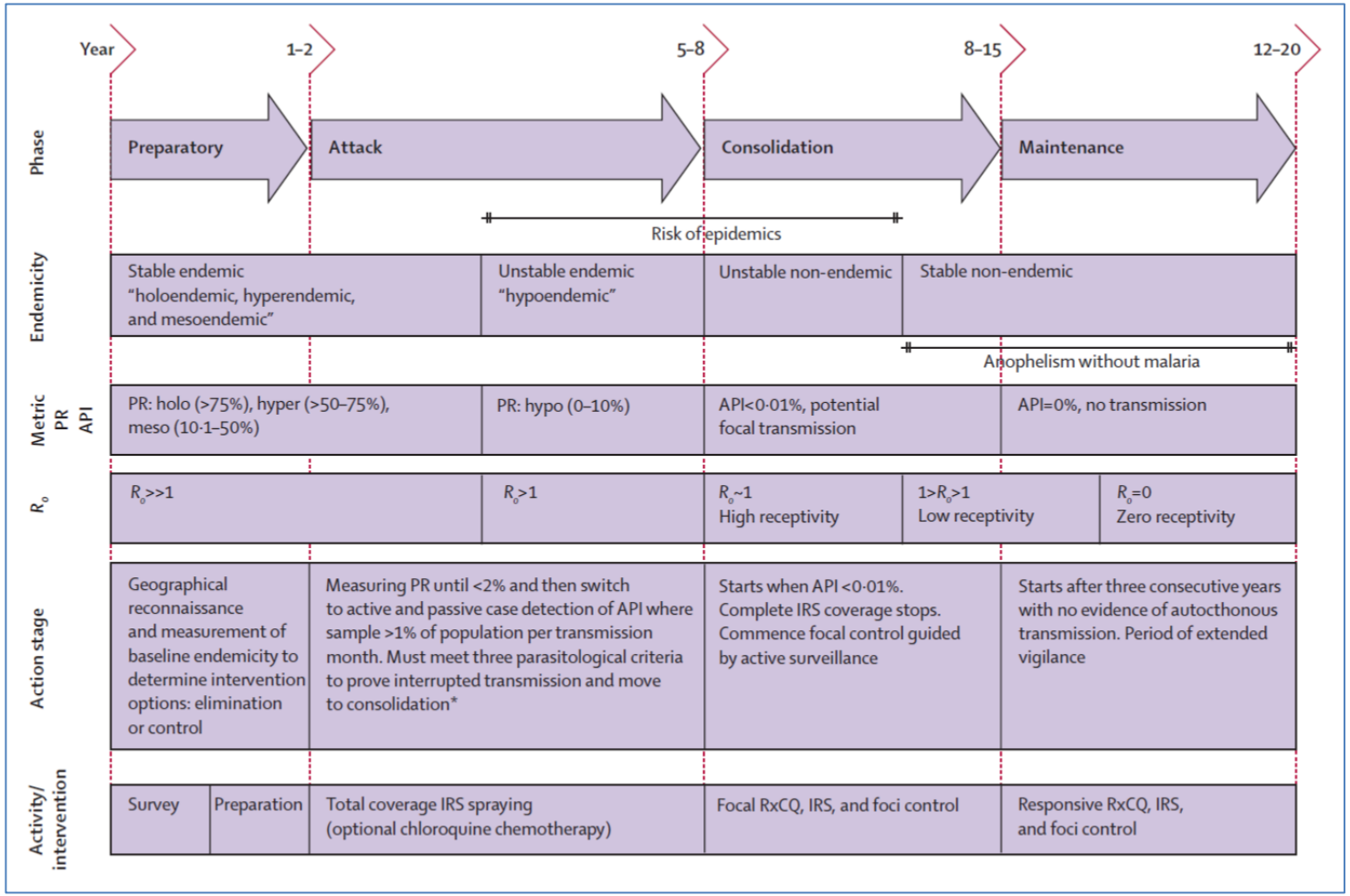


Figure 1.9. Classification for malaria endemicity and the recommended actions. Adapted from reference [111].

To intensify surveillance for malaria, passive case finding has been strengthened with reactive case detection, which is the screening of household members and neighbours within certain radius of index cases detected during passive case detection. This strategy is promoted with the aim to detect high-risk individuals with asymptomatic infection which could further transmit parasites.[112] However, studies suggest the magnitude of effort involved in this approach is not justified due to its laborious workload, and often yielding only few cases. One study suggested targeting hisk risk members of the community (e.g. adult, male, high forest exposure) is more efficient than screening all villagers.[28] Furthermore, asymptomatic cases with positive microscopy results are predominantly in high transmission areas. While in in low endemic settings there can be high proportions of subpatent infections, which are undetected by microscopy or RDT.[80] Therefore, the reactive case detection strategy seems to be ineffective to identify parasites reservoirs, unless performed with higher sensitivity tools.

A different approach has also been considered to accelerate elimination progress by targeting asymptomatic parasite carriers through the implementation of mass drug administration (MDA). This strategy depends on several factors like number of rounds, timing, and frequency of treatment.[113] Studies on MDA are currently being investigated in some countries in the Greater Mekong sub-region (GMS), where malaria transmission is low but multi-drug resistance *P. falciparum* parasites are threatening the sustainability of malaria control. One study in Zambia performed two rounds of MDA with dihydroartemisinin-piperaquine (DP) compared to no mass treatment in low and high transmission areas. All individuals received standard care when suspected with malaria. The endpoint was parasite prevalence after 5 months of follow-up. This study highlighted a large decrease of parasite prevalence among MDA and control groups in both low and high transmission areas. The authors concluded the dramatic reduction in control group might be a result of high vector control coverage, strong surveillance, community diagnosis, and reactive case detection. Nevertheless, the long-term effect of this intervention could still not be explained.[114] Based on different ongoing studies on MDA, a mathematical model predicted a dramatic decrease in malaria prevalence after MDA but to be followed by a rebound to pre-intervention status in the long-term, if elimination is not being established.[115] Until further evidence is available, MDA is not the priority for malaria elimination strategies, and vector control supported by effective case management, is still the most effective form for malaria control sustainability.

Vector control is the mainstay of dramatic reduction in malaria burden in the last two decades. Increasing use of ITNs has averted 450 million cases in Africa, and is considered as

the most important intervention to control malaria.[42] However, this intervention seems to be less effective in the GMS where vectors prefer outdoor and early evening biting.[116] Therefore, ITNs give only limited protection against malaria in this area and are not sufficient to interrupt transmission.[117]

To effectively reduce malaria burden, treatment must be with effective drugs. The next section focuses on previous and current antimalarials and the development of resistance to antimalarials.

1.6 Malaria chemotherapy

Despite the considerable progress on malaria control in the past two decades, emerging resistance to the only current effective antimalarial ACT poses the greatest threat to achieve elimination. *P. falciparum* has a long history of developing resistance to previous antimalarials from chloroquine (CQ), mefloquine (MQ), quinine (QN), amodiaquine (AQ), sulfadoxine-pyrimethamine (SP) and recently artemisinin derivates and piperaquine (Figure 1.10.).[64, 118]. In the past, resistance emerged in one area or sporadically at various times before it spread across countries and continents.[119] The failure to contain the spread of parasites' resistance to the previous mainstays of treatment, CQ and SP, resulted in the deaths of millions of children, particularly in Africa.[120] Therefore, the decline in the efficacy of ACT is extremely worrisome. Although there are several new compounds in the antimalarial pipeline, the development and licensing of new medicines is very complicated, leading to delays before new drugs become available in the market. New chemotherapy should be superior if not similar to current treatment against blood stages confirmed by rapid clinical improvement, only requires single exposure, provides radical cure for hypnozoites stages of *P. vivax* and *P. ovale* spp., and blocks the transmission cycle. This ideal new medicine should meet the minimal acceptable characteristics of the criteria mentioned above, known as Single Exposure Radical Cure and Prophylaxis (SERCaP), and in addition, it should be safe, not contraindicated for pregnant or G6PD-deficient patients, convenient and cost-effective. Nevertheless, in areas where current treatment already show less efficacy, the priority will be different and is to find a drug with activity against resistant strains.[121, 122]

In the absence of effective vaccines and with new drugs not yet licensed, stewardship of ACT is critical to prevent current treatment from failing.[121] Monitoring and evaluating the

current regimen is important to detect early signs of resistance, to identify the spread of resistant parasites and to optimize treatment strategies. Here we will review the therapeutic efficacies of previous and current antimalarials and their development to resistance but concentrating on antimalarial used in Indonesia.

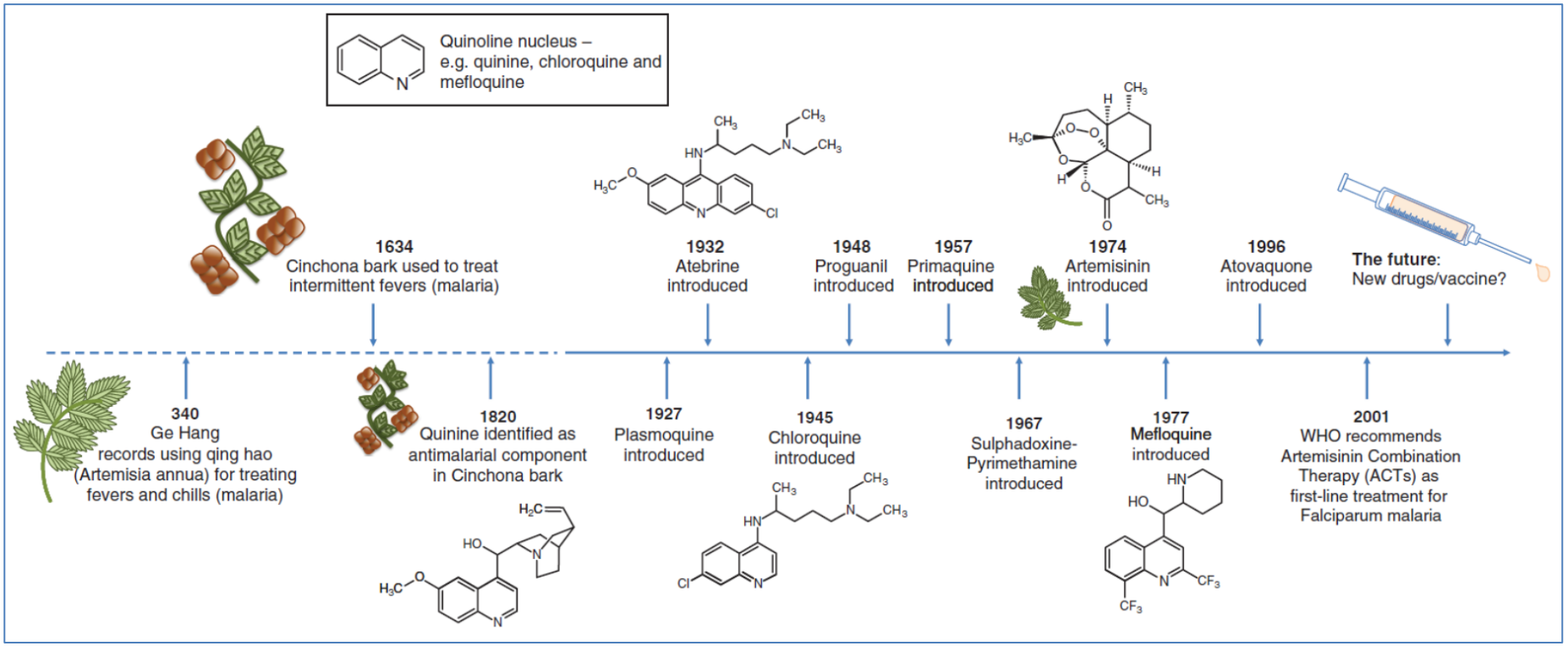


Figure 1.10. Timeline of several antimalarials development. Adapted from reference [123].

1.6.1 Quinine

QN was the first medicine used to treat malaria. It was derived from the bark of Cinchona trees, found in high altitudes of South America in the early 17th century.[124] It was widely used worldwide to treat intermittent fever before the development of synthetic CQ in the 1940s.[123] Quinine alkaloid acts against blood stages[125], but has no antipyretic effect.[126] Despite its early discovery, the active components; QN and cinchonine, were only isolated nearly 200 years later by French chemists, Joseph Caventou and Pierre Pelletier.[123] During colonialism era, the Dutch colony brought Cinchona seeds to Indonesia and established large Cinchona plantations in Java at the end of 19th century, which subsequently made Java the world's biggest producer of QN (providing 97% of world's supply).[127]

In Indonesia, QN is recommended in the national guidelines as the rescue treatment for uncomplicated falciparum and vivax malaria and as an alternative treatment for severe malaria. For uncomplicated malaria, oral QN is given with additional doxycycline or tetracycline for 7 days.[128, 129] *In vivo* studies in North Sumatera between 2006 and 2009 showed that either combination was very effective for uncomplicated malaria, although a high proportion of subjects reported adverse events such as tinnitus.[130-132] Treatment with QN in combination with clindamycin[130, 133, 134] or azythromycin[131, 134-136] was similarly efficacious. In some other parts of the world, QN is still mainly given for severe malaria especially when artemisinin intravenous is not available[128] However, very large multinational studies in Southeast Asia and Africa have both demonstrated significantly lower mortality among children and adults when parenteral artesunate[i] is given compared to parenteral quinine. Therefore, intravenous artesunate is the first choice of treatment for severe falciparum malaria (Figure 1.11).[137-139]

[i] Treatment of severe malaria was given as follows: **Artesunate** 2.4 mg/kg by intravenous or intramuscular injection, followed by 2.4 mg/kg at 12 h and 24 h; continue injection once daily if necessary. **Quinine dihydrochloride** 20 mg salt per kg infused during 4 h, followed by maintenance of 10 mg salt per kg infused during 2-8 h every 8 h (can also be gven as intramuscular injection when diluted to 60-100 mg/mL). **Artemether** 3.2 mg/kg by immediate intramuscular injection, followed by 1.6 mg/kg daily can also be given, when artesunate is not available.

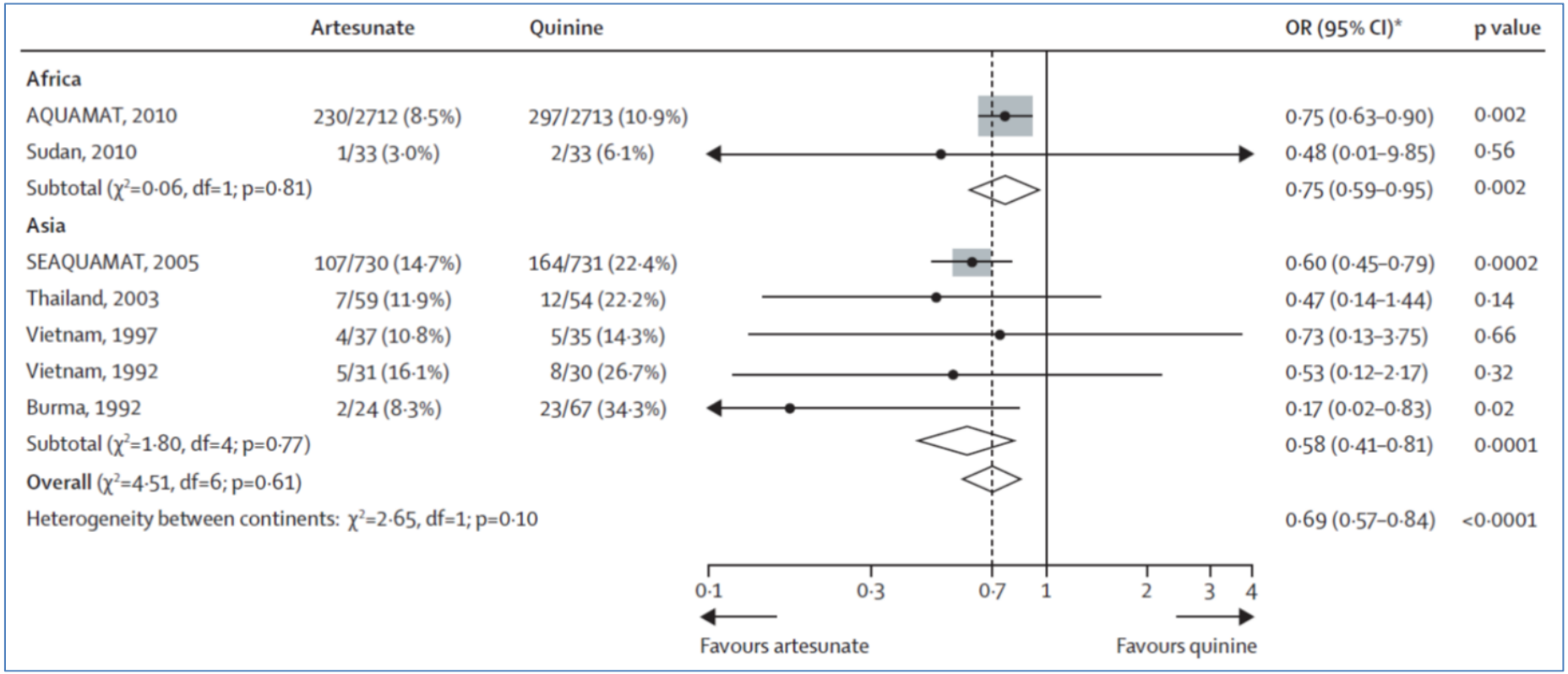


Figure 1.11. Meta-analysis of randomised controlled trials of parenteral artesunate versus parenteral quinine in severe malaria in Asian and African countries. Adapted from reference [5].

1.6.2 Chloroquine

As the Japanese took over Java during World War II, the supply of QN to the rest of the world was halted. The urgent demands of effective drugs for malaria had forced American, British and Australian scientists to collaborate to develop new synthetic antimalarials. An effective but very toxic compound "resochin" discovered few years earlier by Hans Andersag, a German scientist, was one of thousands of compounds tested by this group of scientists. Using additional data from French Vichy physicians working on a resochin derivate named sontochin, a 're-discovery' of a safer and more effective resochin (or later called chloroquine) was made in 1944. CQ became the most widely used antimalarial worldwide, and is still considered the most successful synthetic antimalarial to date.[123, 124, 140] It was cheap, easy to administer, better tolerated and effective against all *Plasmodium* species.[125] However, in the 1960s the first resistance cases emerged in South America and the Thai-Cambodia border. By 1977, CQ resistance arrived in Africa and in 1989 it already spread widely in South America, Asia and Africa with exceptions in some countries (Figure 1.12.).[119, 141]

The first report of *P. falciparum*-resistance to CQ in Indonesia was documented in East Kalimantan in 1974, followed by other reports from various sites in west and east Indonesia. As expected, the overall proportion of resistance quickly increased from 25% prior to 1985 to 54% after 1985, showing a rapid spread of the resistant-parasite (Figure 1.13.).[86, 142] In the 2000s, all studies revealed high level risk of treatment failure[ii] using CQ ranging from 30% to 95%[64, 143-150], consequently the use of CQ as first-line treatment was ended in 2004.[146]

[ii] Treatment failure is defined as an inability to clear malarial parasitaemia or resolve clinical symptoms despite administration of an antimalarial medicine. Treatment failure is not, however, always due to drug resistance, and many factors can contribute, mainly by reducing drug concentrations. These factors include incorrect dosage, poor patient compliance in respect of either dose or duration of treatment, poor drug quality and drug interactions.

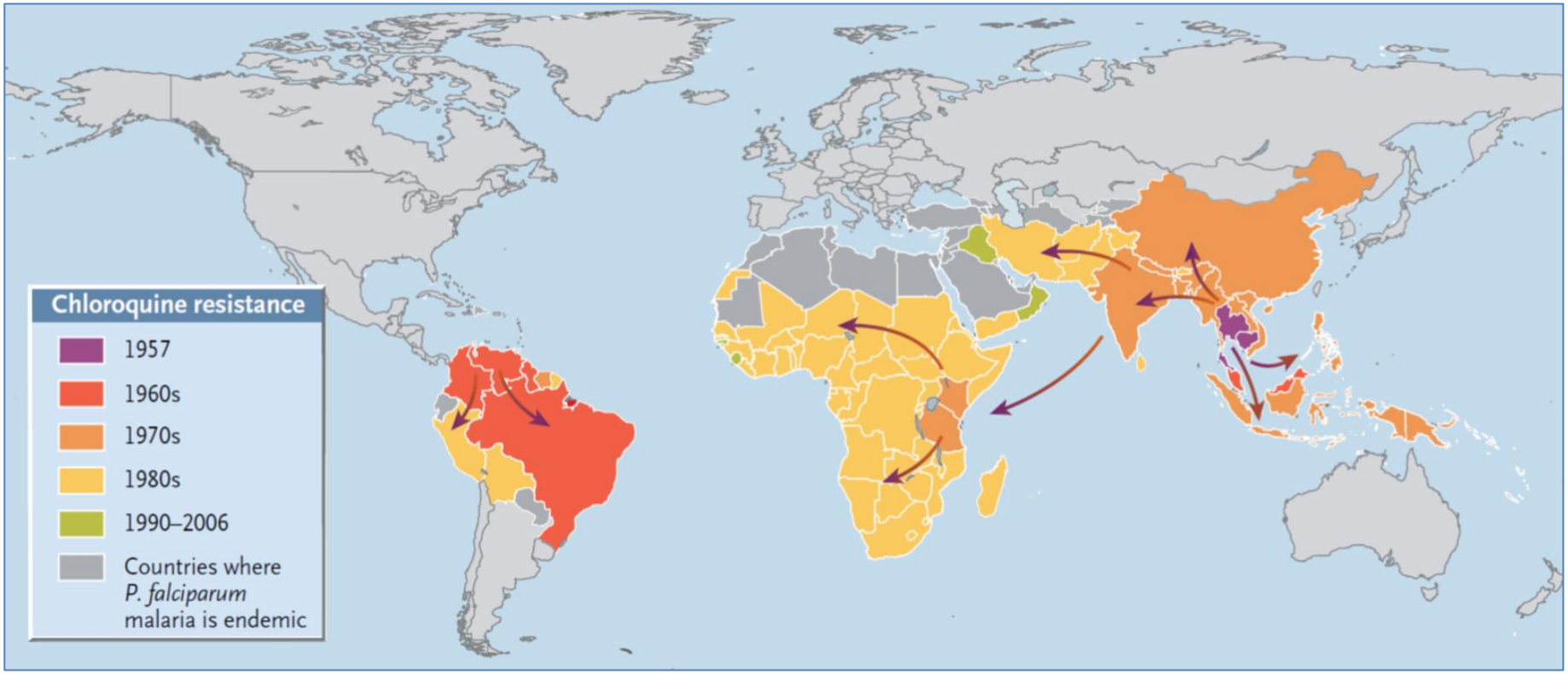


Figure 1.12. The spread of chloroquine resistance. Adapted from reference [141].

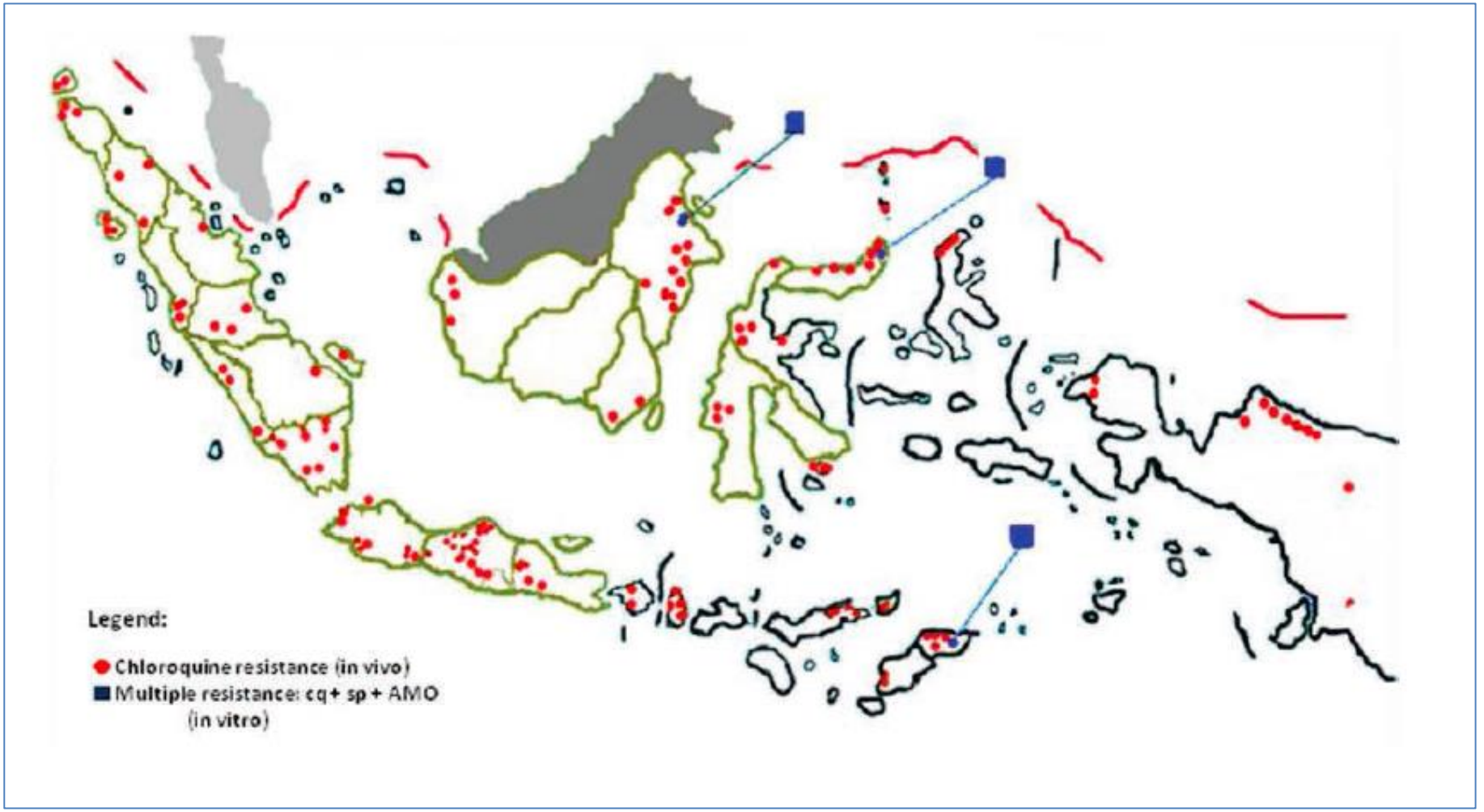


Figure 1.13. Distribution of chloroquine resistance in Indonesia, 1978 – 2003. Adapted from reference [142].

Today, CQ is widely used as the therapy of choice for *P. vivax* infection, usually in a combination with primaquine. It is still the first-line option after over 60 years where vivax is endemic, with exception in some countries in Africa (Algeria, Ethiopia, Mayotte, Somalia, and Sudan) and few others in Southeast Asia (Indonesia and Cambodia), and Oceania (Papua New Guinea, Solomon island, and Vanuatu).[1] Due to the difficulties to define treatment failure in vivax complicated by its hypnozoite inducing relapse, diagnosis of CQ resistance is defined by recurrent parasitaemia within 28 days after therapy with levels of CQ and desethyl-chloroquine above 100 ng/mL.[14] Early evidence of vivax resistance to CQ appeared in Papua New Guinea and eastern Indonesia, with sporadic cases occurred across Malaysia, Myanmar, and Vietnam. Eastern Indonesia has the highest level of resistance with treatment failures > 50%, with lower rates of failure towards western Indonesia.[105, 144, 151-154] However, a later study from South Sumatera in western Indonesia revealed 43% of failures among patients treated with CQ.[147] The national policy was subsequently changed in 2007 to replace CQ with ACT for the treatment of vivax malaria.[128]

There are few data available on CQ and other antimalarials sensitivity to *P. malariae*, *P. ovale* spp. and *P. knowlesi*. Hereafter, there is an assumption of full sensitivity of antimalarials to these parasites.[95, 139, 154] Only 1 study reported resistance of *P. malariae* to CQ. In South Sumatera, 3 out of 28 individuals with *P. malariae* had treatment failure with chloroquine. Two had persistent parasites until day 8 and one had recurrent parasitaemia at day 28, all were showing CQ and desethyl-chloroquine levels > 100 ng/mL at the day of failure.[155] *Ex vivo* susceptibility studies against *P. knowlesi* H strain and *P. knowlesi* field isolates revealed moderate efficacy to CQ with 7-fold higher of IC_{50} in the H strain over the IC_{50} in field isolates (IC_{50} values for H strain vs. clinical isolates, 3.2 nM [2.2 to 4.7] vs. 23 nM [+ 4.8]; R^2=0.86). Surprisingly, in contrast MQ seemed to be ineffective for the laboratory strain and field isolates (IC_{50} values for H strain vs. clinical isolates, 25 nM [7.4 to 81] vs. 26 nM [+ 3.1]; R^2=0.40).[156] Two field studies evaluating the efficacy of CQ *in vivo* in treating *P. knowlesi* patients in Malaysia demonstrated high efficacy with no treatment failures at the end of follow-up (day 28 in Daneshvar study, and day 42 in Grigg study).[157, 158] Although the latter study showed inferiority of CQ when compared to artesunate-mefloquine (AS-MQ) regarding parasite and fever clearance (P <0.0001 and P <0.034).[158]

1.6.3 Sulfadoxine-Pyrimethamine

SP was used by many countries as the second-line treatment for *P. falciparum* infection. The two components act synergistically against folate synthesis, inhibiting dihydropteroate synthetase and dihydrofolate reductase.[159] However, resistance to SP quickly emerged, and regional and intercontinental spread followed (Figure 1.14).[160, 161]

Observation at day 28 following treatment with SP in Indonesia showed evidence of resistance in *P. falciparum* rose from 0% to 83% in 1998, among the highest seen in Indonesia.[105, 144, 150, 162, 163] When CQ was added as a combination, the proportion of treatment failures decreased[146, 149], although in some other studies it remained ubiquitous.[144, 150, 154, 164] SP is now mainly used as intermittent preventive treatment during pregnancy (IPTp), intermittent preventive treatment in infants (IPTi), and in combination with artesunate (AS-SP) for seasonal malaria chemoprevention particularly in sub-Sahel region of Africa[139], with only a few countries deploying antifolates in AS-SP form as first-line treatment for uncomplicated malaria.[1]

Figure 1.14. Spread of sulfadoxine-pyrimethamine resistance. Adapted from reference [165].

1.6.4 Artemisinin-based Combination Therapy

As the efficacy of QN declined due to the extensive use worldwide, the American army research institute (Walter Reed) tried to develop new compounds to protect their soldiers from malaria during the war between the United States and Vietnam. The Vietnamese, on the other hand, turned to China which then initiated the research programmes that resulted in the discovery of artemisinin.[166] Artemisinin or qinghaosu, extracted from the leaves of sweet wormwood (*Artemisia annua*), was discovered in the early 1970s. Its derivatives; artesunate, artemether and dihydroartermisinin (DHA), were also later developed. DHA is the active metabolite, has a greater antimalarial activity than the parent drug.[167] Artemisinin acts against all *Plasmodium* species, is effective against asexual and sexual stages, and thus has transmission blocking advantage. However, liver stages are not affected. This drug is also very potent, it reduces parasite numbers 10^4 fold within the 48 hours erythrocytic cycle.[168, 169]

The artemisinin component is combined with a partner drug with a longer half-life to ensure the clearance of the remaining parasites after rapid reduction by artemisinin, and to prevent the development of resistance.[64, 167, 170] ACTs are the current recommended treatment by WHO for uncomplicated falciparum malaria for all malaria-endemic countries.[64] It is highly effective with few adverse effects.[5] Currently, there are five combinations recommended by WHO: artemether-lumefantrine (AL), artesunate-amodiaquine (ASAQ), dihydroartemisinin-piperaquine (DP), AS-MQ and AS-SP, given over a period of 3 days (See Box 1).[139]

BOX 1 – TREATMENT RECOMMENDATIONS FOR ACTs

Body Weight (kg)	Dose (mg)
Artemether-lumefantrine (AL)	
5 to < 15	20 artemether + 120 lumefantrine
15 to < 25	40 artemether + 240 lumefantrine
20 to < 35	60 artemether + 360 lumefantrine
≥ 35	80 artemether + 480 lumefantrine
Artesunate-amodiaquine (ASAQ)	
4.5 to < 9	25 artesunate + 67.5 amodiaquine
9 to < 18	50 artesunate + 135 amodiaquine
18 to < 36	100 artesunate + 270 amodiaquine
≥ 36	200 artesunate + 540 amodiaquine
Artesunate-mefloquine (AS-MQ)	
5 to < 9	25 artesunate + 55 mefloquine
9 to < 18	50 artesunate + 110 mefloquine
18 to 30	100 artesunate + 220 mefloquine
≥ 30	200 artesunate + 440 mefloquine
Artesunate-sulfadoxine-pyrimethamine (AS-SP)	
5 to < 10	25 artesunate + 250 sulfadoxine/12.5 pyrimethamine
10 to < 25	50 artesunate + 500 sulfadoxine/25 pyrimethamine
25 to < 50	100 artesunate + 1000 sulfadoxine/50 pyrimethamine
≥ 50	200 artesunate + 1500 sulfadoxine/75 pyrimethamine
Dihydroartemisinin-piperaquine (DP)	
5 to <8	20 dihidroartemisinin + 160 piperaquine
8 to < 11	30 dihidroartemisinin + 240 piperaquine
11 to < 17	40 dihidroartemisinin + 320 piperaquine
17 to < 25	60 dihidroartemisinin + 480 piperaquine
25 to < 36	80 dihidroartemisinin + 640 piperaquine
36 to < 60	120 dihidroartemisinin + 960 piperaquine
60 to < 80	160 dihidroartemisinin + 1280 piperaquine
> 80	200 dihidroartemisinin + 1600 piperaquine

All treatment are given once daily for 3 days, except for artemether-lumefantrine twice daily.

ACT was adopted as first-line treatment for uncomplicated *P. falciparum* infection, replacing chloroquine, in 2004. ASAQ was initially recommended for treating uncomplicated falciparum malaria across Indonesia.[86] But in 2007, two ACTs[iii] were recommended by the Ministry of Health of Indonesia, ASAQ for western Indonesia and DP for eastern Indonesia. The change in national policy was also expanded to vivax malaria with ASAQ was being recommended to treat this infection replacing CQ.[128] However, in 2012 DP became the first-line therapy throughout the country. Besides the two combinations, AL is also licensed, and is available in the private sector.

There is regular evaluation and monitoring of ACT efficacy although it is not uniform between endemic areas. One of the earliest studies was conducted in Central Java in 2003 prior to the deployment of ACT. Forty-three patients with uncomplicated *P. falciparum* infection were treated with 3 days of ASAQ, and PCR-corrected adequate clinical and parasitological response (ACPR)[iv] at day 28 revealed low success rate at 80.9%.[171, 172] Follow up studies revealed high efficacy above 98% in different areas[173-175], but two further studies in Papua and Sumatera showed treatment failure above 10%.[176-178] All but one study demonstrated rapid parasite clearance times indicating a good response to artemisinin and treatment failure is likely to be due to AQ.[179] Treatment failure with AQ might be the result of inadequate drug concentration level and cross-resistance with CQ among other factors.[179-181] AQ (a synthetic 4-aminoquinoline) is closely related to chloroquine, but had not previously been used as monotherapy in Indonesia.[182] However, the widespread clinical resistance to CQ has been associated with reduced susceptibility to amodiaquine.[183, 184] Today, this combination is mainly used in African countries and China.[1]

AL is the first fixed-dose treatment available and the most procured ACT for public and private sectors globally[8], with 56 countries using it as first- or second-line treatment.[185] Previous studies in Africa and Southeast Asia (including Indonesia) showed excellent efficacy > 90% with AL[178, 186-194], except in 3 studies from Cambodia and Thailand.[195] One study from Cambodia later confirmed as having artemisinin resistance, and the treatment failures from

[iii] Indonesian Ministry of Health recommendation for uncomplicated falciparum malaria:
Doses for Artesunate-Amodiaquine + Primaquine (3 days + 1 day)
Amodiaquine base (10 mg/kg/day), artesunate (4 mg/kg/day), primaquine (0.75 mg/kg)
Doses for Dihydroartemisinin-Piperaquine + Primaquine (3 days + 1 day)
Dihydroartemisinin (2-4 mg/kg/day), piperaquine (16-32 mg/kg), primaquine (0.75 mg/kg)

[iv] WHO classification for treatment outcomes in *in vivo* study, see Methods section 3.15.

the two studies in Thailand were associated with slow parasite clearance.[195] In Kenya, residual submicroscopic parasitaemia at day 3 was detected in 31.8% patients despite high cure rates and rapid parasite clearance times after treatment with AL or DP. Day 3 positivity was associated with higher risk of reappearance of asexual parasite on day 28 or 42 detected by microscopy.[196] Moreover, pooled analysis of individuals treated with AL showed an increased risk of treatment failure in very young age, high parasitaemia, and low transmission intensity area with emerging resistance.[195] In Uganda, remarkable changes in parasite polymorphisms were seen after increasing use of AL. Within 4 years, there was a reverse trend in the parasites populations from high-level CQ-resistant and lumefantrine-sensitive parasites to CQ-sensitive parasites with decreased sensitivity to lumefantrine.[197]

MQ monotherapy has a long history of utility to treat malaria in the GMS. However, resistance was quickly developed and led to the addition of artesunate to its 3-day regimen.[198, 199] Sadly, resistance to this AS-MQ combination was also soon developed with the first evidence reported from western Cambodia[185, 200], and subsequently from Thailand[185, 201] and Myanmar[185, 202]. Despite the significant failures, the declining efficacy was full responsibility of mefloquine resistance and not attributed by the artemisinin component.[198] Dihydroartemisinin-piperaquine (DP) later replaced AS-MQ as the first line treatment in 2008 for Cambodia and in 2015 for Thailand. Interestingly, repeat studies on AS-MQ in Cambodia between 2014 and 2017 revealed 100% efficacy suggesting that AS-MQ has regained its efficacy after removal of MQ pressure to parasites.[203]

DP is considered by many to be the best ACT due to the long serum half-life of the partner drug, resulting in post-treatment prophylaxis.[186] Various studies in Africa and Asia have shown superior efficacy of DP compared to the other most used ACTs; ASAQ and AL.[179, 186, 187, 190, 204-208] One study of DP from west Indonesia showed 100% ACPR[209], with a small proportion of treatment failures (0% to 4.8%) in studies from east Indonesia.[176, 193, 210-213] A major determinant of treatment failures was plasma concentration of piperaquine below 30 ng/ml on day 7.[211] Prior to 2010, only one study from Rwanda (to our knowledge) reported treatment failure exceeding 10% after treatment with DP which necessitated a changes in antimalarial policy according to the WHO guidelines.[185, 214] In Cambodia, the efficacy of DP has declined rapidly. Prior to policy changes to DP in 2008, the efficacy of DP in Pursat was 98.2% in 2005[215] before falling to 89.3%[216] in 2010 and to 54%[118] in 2013. Recrudescent parasites showed high piperaquine level in the plasma at day 7 and increased piperaquine IC_{50}, implying sufficient exposure of partner drug to the parasites and also suggesting that there is an

emergence of resistance to artemisinin and also to piperaquine.[118] All studies of DP efficacy in Indonesia demonstrated excellent efficacy.[176, 193, 209-212, 217, 218] WHO recommends to conduct therapeutic efficacy studies at least once every 24 months to allow early detection of changes in antimalarial efficacy.[203] Figure 1.15. and table 1.1. summarised ACTs efficacies for uncomplicated *P. falciparum* malaria in Indonesia.

There are limited studies evaluating the efficacy of ACTs to other *Plasmodium* species. Most studies were done on *P. vivax* showing excellent efficacy with superiority using DP compared to AL, ASAQ and AS-SP, but similar efficacy to AS-MQ.[219] In Indonesia, studies on *P. vivax* showed high efficacy[193, 211, 220-224] except in one study from Papua with 48% recurrences after ASAQ.[176] Nevertheless, the cause of infection could not distinguish between recrudescence, relapse or reinfection.[176] ACT was also shown to be effective in limited studies on *P. malariae* and *P. ovale*.[219] In other limited studies on *P. knowlesi*, AS-MQ, AL and DP were successful to treat uncomplicated knowlesi malaria.[77, 158, 225-227]

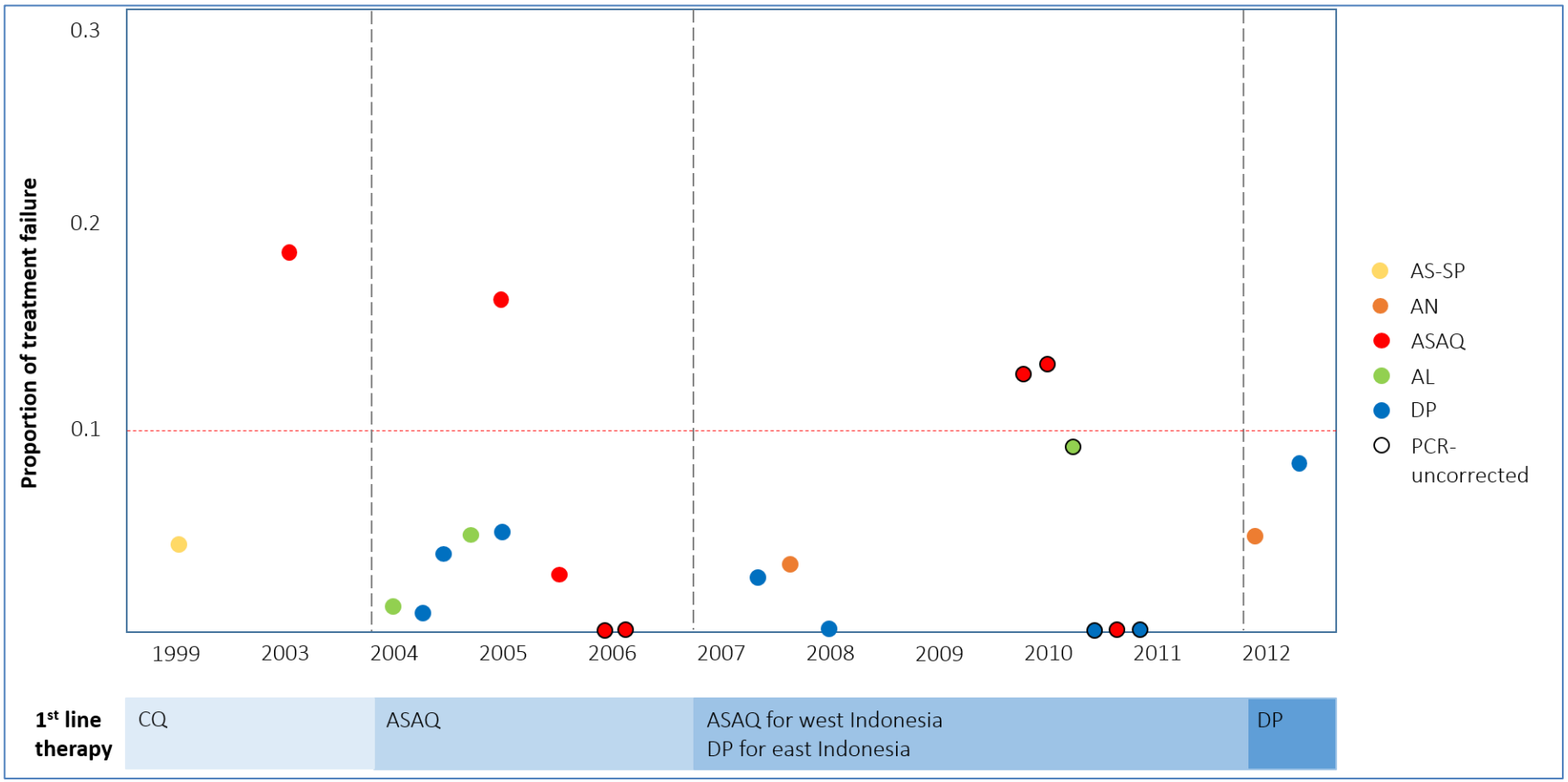


Figure 1.15. Proportion of treatment failures in various *in vivo* studies using five different ACTs across Indonesia. (AS-SP, Artesunate-Sulfadoxine-Pyrimethamine; AN, Artesunate-Naphtoquine; ASAQ, Artesunate-Amodiaquine; AL, Artemether-Lumefantrine; DP, Dihydroartemisinin-Piperaquine; Treatment failure > 0.1 is the cut-off recommended by WHO for new treatment policy). Based on data from references [162, 171, 173, 174, 176, 193, 194, 211, 212, 228, 86, 177, 178, 209, 210, 217, 218]

Table 1.1. Summary of *in vivo* studies on ACTs efficacies for treatment of uncomplicated *P. falciparum* infection across Indonesia

Year	Sites	No.of samples	Follow-up	Mean PCT (hours)	Day-3 positive* (%)	ACPR (%)	PCR-corrected	Ref
Artesunate-Sulfadoxine-Pyrimethamine (AS-SP)								
1999	Papua	53	28	33.6	0	95.6	Yes	162
Artesunate-Amodiaquine (ASAQ)								
2003	Cenral Java	43	28	24	13.9	80.9	Yes	171
2005	Papua	148	42	48	0	84	Yes	176
2005-6	West Sumba	103	28	48	12.6	97	Yes	174
2006	North Sumatera	121	28	47	9	100	No	173
2006	North Sumatera	116	28	48	NA	100	No	228
2010	South Sumatera	23	28	NA	13	87	No	177
2010	North Sumatera	140	42	NA	NA	86.4	No	178
2010-11	Central Java	9	28	48	0	100	No	217
Artemether-Lumefantrine (AL)								
2004	East Sumba	79	42	32.3	0	98.3	Yes	194
2004-5	Papua	290	42	48	0	95.3	Yes	193
2010	North Sumatera	140	42	NA	NA	90.7	No	178
Dihydroartemisinin-Piperaquine (DP)								
2004-5	Papua	**515**	**42**	48	NA	98.9	Yes	211
2004-5	Papua	**289**	**42**	48	3	95.9	Yes	193
2005	Papua	**151**	**42**	48	0	95.2	Yes	176
2007-8	Papua	**122**	**42**	25.5	NA	97.3	Yes	212
2008	Lampung	**349**	**42**	NA	NA	100	Yes	209
2010-11	Kalimantan & Sulawesi[a]	**119**	**28**	38.4	0.84	100	No	210
2010-11	Central Java	**37**	**42**	48	0	100	No	217
2012	5 provinces	97	42	NA	10.3	91.9	Yes	218
Artesunate-Naphtoquine (AN)								
2007-8	Papua	121	42	28	NA	96.3	Yes	212
2012	5 provinces	71	42	NA	1.4	95.1	Yes	218

* Presence of asexual *P. falciparum* parasite detected by microscopy

[a] Central Kalimantan, West Kalimantan, Central Sulawesi

1.6.5 Atovaquone

Atovaquone, in a fixed-dose combination with proguanil (Malarone), is used as a chemoprevention especially for travelers to malaria-endemic countries[229-231]. Although the extent use of this drug is limited to travelers, several cases of treatment failure have been reported.[232, 233] Resistance to atovaquone alone, tested in rodent malaria parasite *Plasmodium berghei,* develop quickly, and has been associated with point mutation at codon 268 in *cytochrome b*. Resistant parasites survive drug pressure by atovaquone, however they are unable to complete their development in mosquito phase, therefore are not transmissible. Further experiments to determine similar phenomenon in *P. falciparum* infections suggested that these human malaria parasites were also unable to transmit atovaquone-resistant mutations, and might give some advantage for its use for malaria control.[234]

1.6.6 Antibiotics

In vitro and *in vivo* studies have shown antimalarial activity of several antibiotics classes including tetracyclines (tetracycline and doxycycline)[235-238], macrolides (erythromycin, azythromycin)[239-242], and fluoroquinolones (ciprofloxacin)[243, 244]. These antibiotics are slow acting but have potent antimalarial effect against the apicoplast, a phenomenon referred to as "delayed death".[245] Therefore, antibiotic is not ideal to be used as monotherapy but should be given in conjunction with rapid acting drugs such as artemisinin or quinine.[246] Doxycycline monotherapy nevertheless has been shown to be effective as malaria prophylaxis, although it is not recommended for pregnant women and children < 8 years of age.[247]

1.6.7 Primaquine

Although artemisinin has an impact on gametocyte carriage and thus reduces mosquito infectivity[248], a single dose of primaquine is still recommended as a gametocytocidal in addition to ACT treatment particularly in low transmission areas.[5] Primaquine is mainly used as radical cure for treatment of *P. vivax*[249], but is also beneficial to block transmission of *P. falciparum* infection.[250] Single doses of primaquine either at 0.25 mg/kg or at 0.75 mg/kg (WHO recommendation[250]) have both shown increased in gametocyte clearance in children in Uganda with no difference in the risk of developing anemia, a known risk due to primaquine-

induced haemolysis in individiuals with glucose-6-phosphate dehydrogenase (G6PD) deficiency.[251] Similar evidence was seen in a study in Indonesia where the addition of primaquine to ACT as recommended in national policy contributed to 16.6 times lower in gametocyte prevalence at day 14 compared to placebo.[209] In the context of radical treatment, the optimal dosage of primaquine has long been debated due to its safety concerns.[249] It is currently recommended to be given at dose 0.25-0.5 mg/kg/day for 14 consecutive days.[139] The efficacy of primaquine in preventing relapse at lower dose (0.25 mg/kg) was comparable to higher dose (0.5 mg/kg). In 1 year follow-up, 11.5% and 9.1% of patients had relapse after receiving AL and DP plus 14-day of lower dose of primaquine, compared to 6% of relapse in patients treated with DP plus 14-day of higher dose primaquine.[221] Low prevalence (3.3%) of G6PD deficiency with "mild-type" variant from findings in North Sumatera[220] suggested that primaquine at certain low dose[v] can be deployed safely without prior testing for deficiency.[250]

1.7 The fall of ACTs

ACT has been used in Cambodia and Vietnam for over 20 years, and the first report of a potential emergence of resistance to artemisinin came from Pailin, western Cambodia in 2009.[252] Noedl and Dondorp expressed their concerns about the declined sensitivity to artemisinin reflected by slow parasite clearance. Patients with uncomplicated falciparum were treated with artesunate monotherapy (4 mg/kg/day[252] or 2 mg/kg/day[253] for 7 days) or artesunate (4 mg/kg/day for 3 days) plus mefloquine (15 mg/kg at day 3 and 10 mg/kg at day 4[253]). A markedly slower parasite clearance time with a small proportion of recrudescences were observed, however they were not necessarily associated.[253] Artemisinin resistance was defined (see Box 2[203]) and follow-up studies also observed similar evidence in Cambodia[254, 255], Thailand[120, 255], Vietnam[255-257], Myanmar[255, 258], Laos[203] and China[259]. Proportion of patients with day 3 positivity have also been increasingly noted over the years, suggesting a rapid spread or a takeover of this resistant parasite in the population.[120, 216, 252, 253, 255, 259-261] However, slow parasite clearance does not necessarily cause ACT failure. ACT failure unaccompanied by slow rate clearing is likely to occur when the partner drug fails to eliminate residual parasites.[171, 176, 198] As long as the partner drug of ACTS remain effective, ACTs are still the most

[v] Primaquine was given at dose of 0.25 mg base/kg (or 15 mg for > 40 kg) for 14 days.[220]

effective antimalarial for uncomplicated falciparum malaria despite the delay in parasite clearance in some areas of the GMS.[203] Nevertheless, reduced sensitivity to artemisinin allows pressure on the partner drug, and may facilitate the emergence of partner drug resistance.[262] Unfortunately, as the proportion of patients with artemisinin resistance increases, there is a greater pressure on the partner drugs creating *de novo* resistance which may subsequently result in catastrophic ACT failures across the GMS (Figure 1.16.)[203]

However, there is an argument that prolonged parasite clearance should not be used solely to define artemisinin resistance. In western Cambodia where proportion of patients with day 3 positivity has significantly increased over the years, longer course of treatment with 7 days of artesunate monotherapy (4 mg/kd/day) still provided 94.7% efficacy.[263] As well as in a study where 26.9% of patients had day 3 positivity after artemisinin monotherapy later on showed 100% adequate clinical and parasitological responses at day 28.[258] ACT failures only occurred where there is both resistance to artemisinin and the partner drug. Therefore, the classical definition of resistance which usually includes treatment failure at days 28 of follow-up is currently not being applied in the current definition of artemisinin resistance.[264]

BOX 2 – **DEFINITION OF ARTEMISININ RESISTANCE**

There is a long debate whether delay in parasite clearance after treatment with artemisinin should be defined as 'artemisinin resistance'. WHO stated there is no evidence that full resistance have emerged, but current clinical evidence with the presence of *P. falciparum* kelch 13 (*pfk13*) mutations strongly suggests evidence of partial resistance. However, definition below is subject to change based on new evidence.

Clinical artemisinin resistance

It is defined as delayed parasite clearance, it represents a partial/relative resistance that has thus far only affected ring-stage parasites.

Suspected endemic artemisinin resistance

- ≥ 10% of patients with a half-life of the parasite clearance slope > 5 hours after treatment with ACT or artesunate monotherapy; or
- ≥ 5% of patients carrying K13 resistance-confirmed mutations (listed in Table 1.5. – see Section 1.8.1.); or
- ≥ 10% of patients with persistent parasitaemia by microscopy at 72 hours (± 2 hours; i.e., day 3).

Confirmed endemic artemisinin resistance

- ≥ 5% of patients carrying K13 resistance-confirmed mutations, all of whom have been found to have either persistent parasitaemia by microscopy on day 3 or a half-life of the parasite clearance slope ≥ 5 hours after treatment.

The mechanisms of antimalarial drug resistance are mediated by two processes: drug selection of resistant parasites; and the spread of those resistant alleles to other individuals. The appearance of *de novo* mutations is initially only relevant in the individuals harboring these mutants and the use of drugs contributes to the selection of resistant parasite strains.[265] In Cambodia, long-term use of artemisinin monotherapies is among other reasons including substandard drugs and subtherapeutic doses of treatment that have contributed to the increased drug pressure. Strategies to minimize drug pressure, including a ban of monotherapy use, have been implemented, yet it remains a challenge.[165] Once resistant parasites survive the host immune response, which is usually less developed in low-transmission areas, resistant parasites transfer to a new individual and subsequently spread within and between populations.[265]

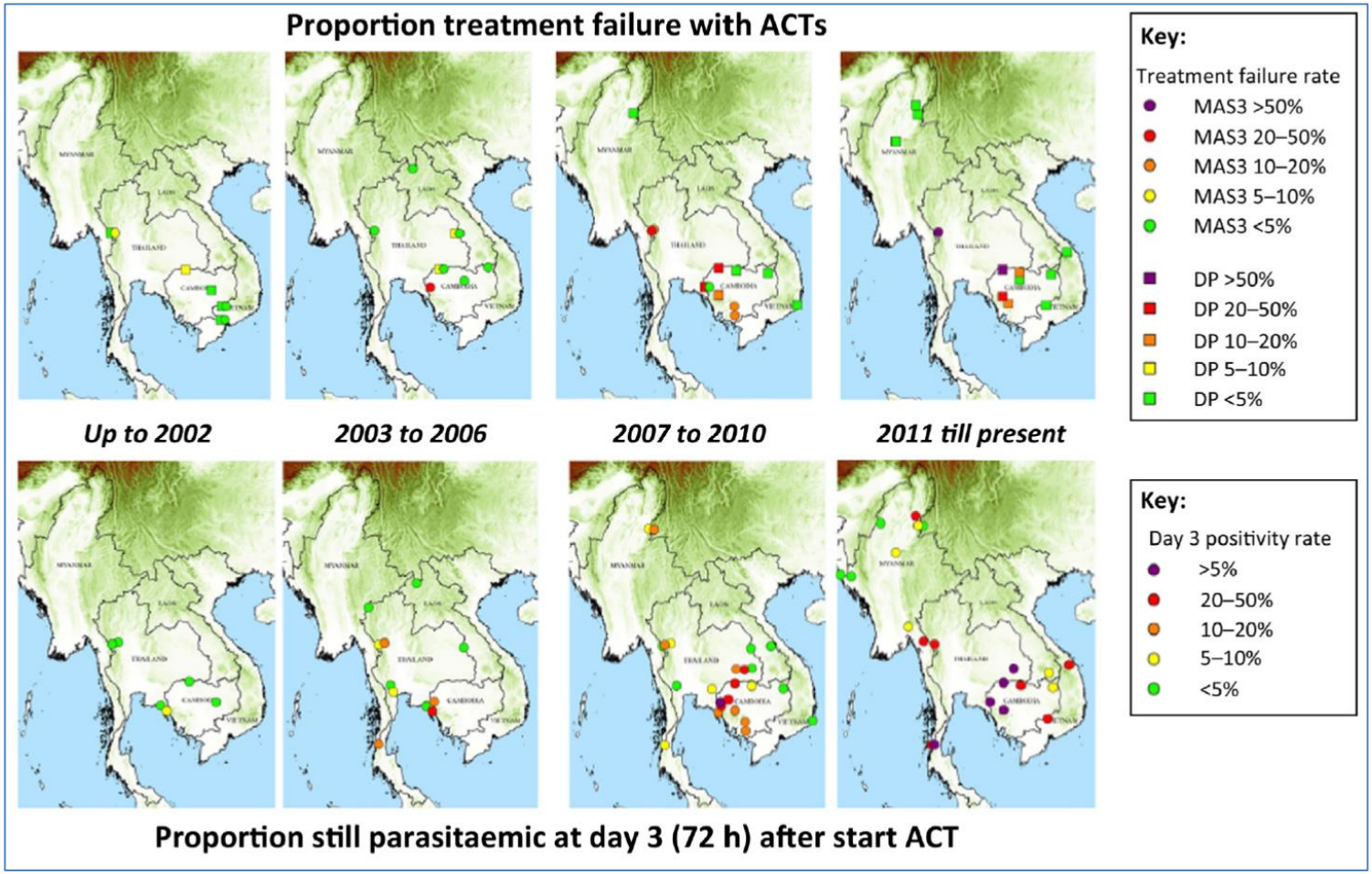


Figure 1.16. Regions where ACTs failing have been reported. Adapted from reference [266].

1.8 Molecular markers for drug resistance

Molecular markers are an important tool to detect and monitor the presence of antimalarial resistance. Their significant implication is to geographically map the extent of resistant-parasites, thus enabling strategies for their control and elimination to be applied before an inevitable increase in the disease burden occurs.[267] Different markers have been used to identify antimalarial resistance including a molecular marker for artemisinin.[268]

Detection of mutations in the *pfcrt* gene has been used to identify CQ resistance.[269] There are 15 polymorphisms in the gene linked to the resistance, but KT76 mutation is the essential determinant to confer resistance to chloroquine in *P. falciparum* malaria.[270] Codons 72 – 76 are particularly interesting and contribute to several resistant variants related to their geographical origins. The haplotype encoding amino acids CVMNK at that region represents wild-type parasites, while the CVIET haplotype is associated with resistant isolates from Africa and Southeast Asia, and SVMNT is linked to chloroquine resistant strains in South America and some parts of Asia.[271] However, the degree of CQ resistance is modulated by additional mutations from other *P. falciparum* genes such as P-glycoprotein homolog (Pgh-1)-encoding gene, *pfmdr1*.[181, 272] Mutations at codons 86, 184, 1034, 1042 or 1246 of the *pfmdr1* gene have been associated with CQ resistance, and a higher risk of CQ treatment failure was associated with the presence of parasites harboring the N86Y mutation.[272] *Pfcrt* and *pfmdr1* polymorphisms have also been correlated to AQ resistance.[181, 272-274] Level of resistance is highly dependent upon the contributions of SVMNT haplotype[274], while CVIET alone is not sufficient to confer AQ resistance.[273] Interestingly, evidence has shown that AQ and AL select different alleles at codon 86, 184 and 1246 of *pfmdr1* , with amodiaquine positively selects for haplotype YYY while AL selects the opposite NFD.[273] Molecular surveillance at different time points in Uganda confirmed genotypic changes in the parasite population with decreasing trends in aminoquinolone-resistant polymorphisms in *pfcrt* (76T) and *pfmdr1* (86Y/184Y/1246Y) after increasing use of AL in the country.[197] Similar trends in the resistance markers were also observed in a longitudinal study implementing AL and DP for treatment over 5 years with greater changes observed in the AL group.[275] Consistent to the polymorphic changes, the impact on clinical efficacy was shown in a study on ASAQ versus AL showing less risk of recurrence (P<0.001) and recrudescence (P<0.006) in the ASAQ group.[276] Mutation at N1042D of *pfmdr1* has shown a strong association with quinine, mefloquine and artemisinin.[181] Treatment failure with mefloquine and artemether-lumefantrine has also been

demonstrated with increased copy number of *pfmdr1* in studies from Asia, although clinical failure has not been seen in Africa.[199, 261, 277] In contrast, deamplification of *pfmdr1* has been associated with reduced response to piperaquine.[216]

Various investigations in west and east Indonesia identified wide distribution of mutant alleles of *pfcrt* and *pfmdr1* genes (Figure 1.17.), with high prevalence of K76T mutation of *pfcrt* but more heterogeneity in the proportion of 86Y and 1042D mutant alleles of *pfmdr1* (Table 1.2.). The high frequency of mutant alleles is consistent with the high treatment failure of chloroquine observed in *in vivo* studies.[278] Studies from east Indonesia also revealed parasites carrying SVMNT and CVIET haplotypes, although the latter was in a much smaller proportion.[279-282] The SVMNT haplotype is similar to that found in Papua New Guinea and East Timor where chloroquine and amodiaquine were both widely used, and aminoquinolone resistance has previously been described.[283-286]

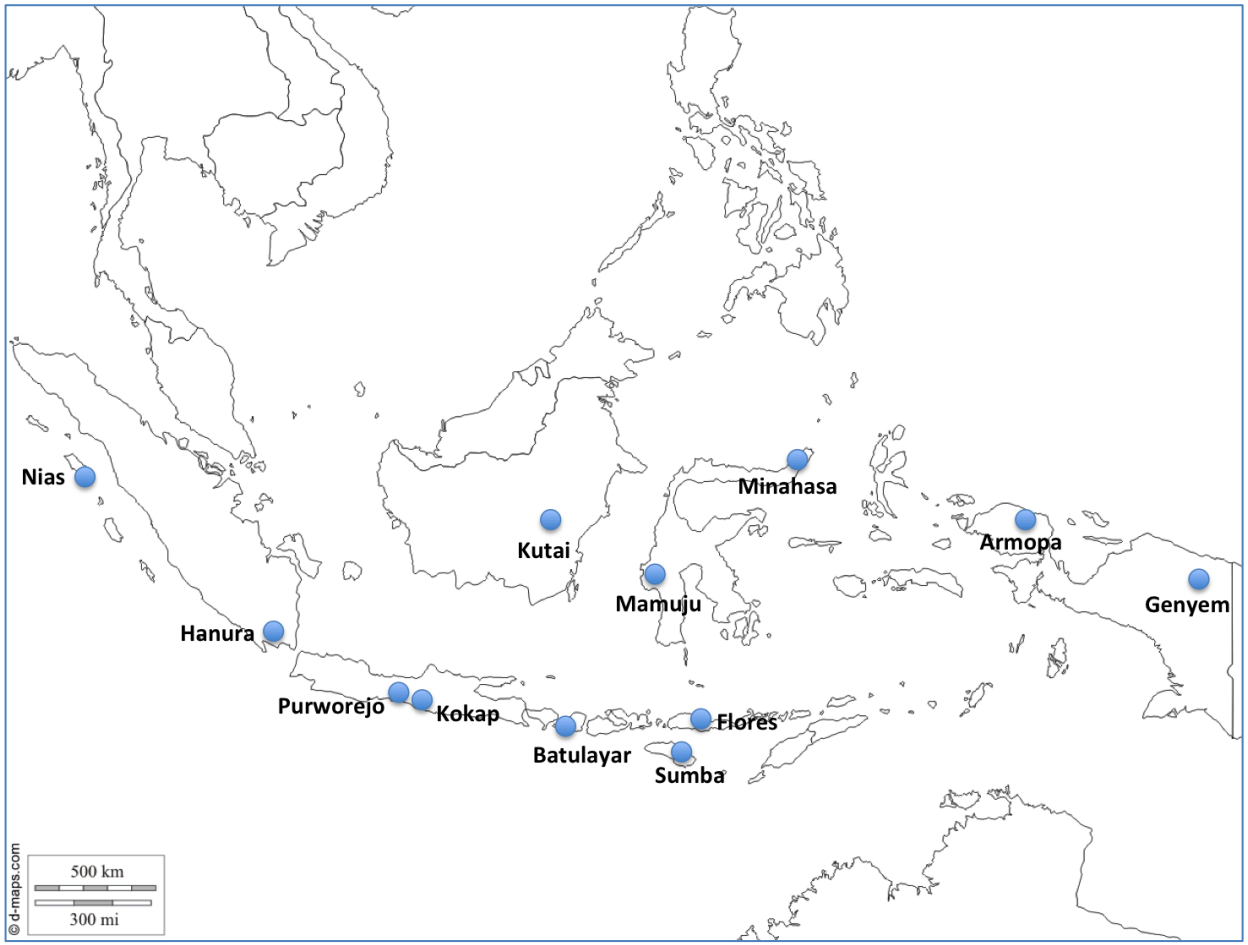


Figure 1.17. Sampling sites across Indonesia for studies of drug resistance markers of *P. falciparum*

Table 1.2. Frequency distribution of mutant alleles of *pfcrt* and *pfmdr1* genes from *P. falciparum* isolates in various sites in Indonesia after 2000

Year	Area	No. of samples	Proportion with mutant alleles, % (*n*)					Ref
			Pfcrt	*Pfmdr1*				
			76T	86Y	1034C	1042D	1246Y	
2000	Purworejo	111	99 (111)	92 (111)	0 (111)	4.5 (111)	0 (111)	[278]
2002	Batulayar	48	97.9 (48)	35.4 (48)	NA	NA	NA	[279]
2003	Kokap	35	94.7 (19)	100 (32)	0 (32)	0 (32)	0 (24)	[278]
2003	Hanura	48	100 (25)	100 (33)	0 (33)	0 (33)	0 (33)	[278]
2003	Nias	20	100 (19)	100 (19)	0 (19)	0 (20)	0 (19)	[278]
2003	Kutai	28	100 (19)	100 (28)	0 (19)	5.2 (19)	0 (19)	[278]
2003	Mamuju	25	100 (16)	62.5 (16)	0 (22)	13.6 (22)	0 (24)	[278]
2003	Minahasa	41	94.7 (19)	94.7 (19)	0 (24)	13 (3)	0 (24)	[278]
2003	Armopa	21	88.2 (17)	26.6 (15)	0 (17)	0 (15)	0 (17)	[278]
2003	Genyem	93	89.1 (93)	27.3 (93)	0 (93)	19.3 (93)	0 (93)	[278]
2003	Flores	21	100 (19)	16.6 (19)	0 (19)	89.4 (9)	0 (19)	[278]
2004	Nias	106	99 (101)	31.4 (102)	NA	2 (102)	0 (102)	[107]
2005	Sumba	103	97.1 (103)	20.4 (103)	NA	16.5 (103)	0 (103)	[174]
2007	Sumba*	213	92.9 (213)	41.9 (131)	0 (191)	72.8 (191)	0 (191)	[106]
2007	Sumba**	231	84.9 (174)	42.8 (63)	0 (191)	53.3 (180)	0 (191)	[106]
2010	Minahasa	44	100 (44/44)	88.2 (45/51)	NA	NA	NA	[282]

Legend: *wet season; **dry season

Mutations in the dihydrofolate reductase (*dhfr*) gene are known to be responsible for pyrimethamine resistance with a point mutation at codon 108 to be sufficient enough to confer resistance.[287] This point mutation can arise spontaneously and frequently in a rapid manner after only short exposure to pyrimethamine.[161] In Southeast Asia where a high level of pyrimethamine resistance has been documented, four amino acid changes have been commonly identified, while only one to three mutations have been observed in Africa.[288] Double mutations in *Pfdhfr* gene encompassing 108N mutant with either 51I or 59R allele confer greater levels of pyrimethamine resistance[287] which usually manifests in slower

parasite clearance times. Triple-mutation at codons 108, 51 and 59 results in high treatment failure and four mutations with additional mutation at codon 164 leave the parasite untreatable.[272, 288, 289] Genetic studies revealed despite multiple origins of low-level resistance, three point mutations of *dhfr* which confer full resistance to SP appeared to only have two origins, in South America and Southeast Asia.[288, 290, 291] SP resistance seems to originated in these two regions and the spread followed the pattern of chloroquine-resistant parasites.[161]

Five amino acid changes have been described in sulfonamide resistance: 436A/F, 437G, 540, 581G, and 613T/S.[292] A single mutation in the dihydropteroate synthetase (*pfdhps*) gene can confer a level of sulfonamide resistance, however double mutations at 436F and 613S/T or point mutation at 581G contribute much higher levels of resistance.[287] Triple mutant alleles in the *pfdhps* conferring resistance involving codons 437, 540, and 436 or 581 are prevalent in Southeast Asia, while in West and Central Africa parasites harboring a single mutation at codon 437 or in combination with mutant allele 436A/F are the most common. In East Africa, wild type parasites and combinations of 437 and 540 mutations are well established.[292]

The prevalence of polymorphisms in the *pfdhfr* and *pfdhps* genes (Table 1.3. and 1.4.) in Indonesia is less frequent compared to that observed in the *pfcrt* and *pfmdr1* genes. Double mutations in the *pfdhfr* gene were observed with the most common pair identified being 108N and 59R, which accounted for approximately 30% of all samples.[106, 108, 174] Mutation in the the *pfdhps* gene is less common with a single mutation at 437G as the most common, followed by mutation at 540E. Triple mutations reported in Southeast Asian countries were not seen in the studies in Indonesia.[292]

Table 1.3. Frequency distribution of mutant alleles of *pfdhfr* gene from *P. falciparum* isolates in various sites in Indonesia after 2000

Year	Area	No. of samples	Proportion with *Pfdhfr* mutant alleles, % (*n*)							Ref
			16V	50R	51I	59R	108N	108T	164L	
2000	Purworejo	111	29 (111)	0 (111)	0 (111)	46 (111)	51 (111)	27 (111)	0 (111)	[278]
2003	Kokap	35	33 (27)	0 (27)	0 (27)	33 (27)	41 (27)	67 (27)	0 (27)	[278]
2003	Hanura	48	0 (32)	0 (32)	0 (32)	63 (32)	78 (32)	34 (32)	0 (32)	[278]
2003	Nias	20	0 (17)	0 (17)	0 (17)	75 (16)	94 (17)	0 (17)	0 (17)	[278]
2003	Kutai	28	0 (18)	0 (18)	0 (18)	0 (18)	56 (18)	5 (18)	0 (18)	[278]
2003	Mamuju	25	9 (22)	0 (22)	0 (22)	27 (22)	27 (22)	73 (22)	0 (22)	[278]
2003	Minahasa	41	0 (20)	0 (20)	0 (20)	10 (20)	55 (20)	75 (20)	0 (20)	[278]
2003	Armopa	21	0 (19)	0 (19)	0 (19)	68 (19)	68 (19)	0 (19)	0 (19)	[278]
2003	Genyem	93	0 (93)	0 (93)	0 (93)	58 (93)	79 (93)	0 (93)	0 (93)	[278]
2003	Flores	20	10 (20)	0 (20)	0 (20)	35 (17)	0 (20)	90 (20)	0 (20)	[109]
2004	Nias	109	0 (69)	0 (69)	0 (69)	52.2 (69)	82.5 (69)	0 (69)	0 (69)	[107]
2005	Sumba	103	0 (103)	0 (103)	0 (103)	37.8(103)	87.4(103)	0 (103)	NA	[174]
2007	Sumba*	213	0 (99)	0 (99)	0 (99)	25.6 (99)	31.5 (99)	0 (99)	0 (99)	[106]
2007	Sumba**	231	0 (73)	0 (73)	0 (73)	25.4 (73)	24.7 (73)	0 (73)	0 (73)	[106]

Legend: *wet season; **dry season

Table 1.4. Frequency distribution of mutant alleles of *pfdhps* gene from *P. falciparum* isolates in various sites in Indonesia after 2000

Year	Area	No. of samples	Proportion with *Pfdhps* mutant alleles, % (*n*)					Ref
			436F/A	437G	540E	581G	613S/T	
2000	Purworejo	111	0 (111)	35.3 (111)	26.5 (111)	0 (111)	0 (111)	[278]
2003	Kokap	35	0 (28)	3.57 (28)	3.57 (28)	0 (28)	NA	[278]
2003	Hanura	48	0 (48)	20.8 (48)	0 (48)	0 (48)	0 (48)	[278]
2003	Nias	20	0 (20)	0 (20)	0 (20)	0 (20)	0 (20)	[278]
2003	Kutai	28	0 (28)	17.8 (28)	0 (28)	0 (28)	0 (28)	[278]
2003	Mamuju	25	0 (25)	4 (25)	0 (25)	0 (25)	0 (25)	[278]
2003	Minahasa	41	0 (40)	22.5 (40)	2.5 (40)	0 (40)	0 (40)	[278]
2003	Armopa	21	0 (21)	23.8 (21)	4.7 (21)	0 (21)	0 (21)	[278]
2003	Genyem	93	0 (93)	25 (93)	21.7 (93)	0 (93)	0 (93)	[278]
2003	Flores	20	0 (20)	0 (20)	0 (20)	0 (20)	0 (20)	[109]
2004	Nias	109	0 (69)	1.18 (69)	1.18 (69)	0 (69)	0 (69)	[107]
2005	Sumba	103	0 (103)	3.9 (103)	0.9 (103)	0 (103)	0 (103)	[174]
2007	Sumba*	213	0 (92)	0 (92)	0 (92)	0 (92)	0 (92)	[106]
2007	Sumba**	231	0 (92)	2.2 (92)	0 (92)	0 (92)	0 (92)	[106]

Legend: *wet season; **dry season

Although single copy number of *pfmdr1* has been associated with decreased piperaquine susceptibility, there was no validated marker to identify piperaquine resistance specifically.[216] Initial work to predict treatment failure after DP was the development of piperaquine survival assay (PSA). Culture-adapted and fresh *P. falciparum* isolates were exposed to 200 nM piperaquine for 48 hours and then monitored for 24 hours. PSA survival rates $\geq$ 10% was associated with higher risk of recrudescence.[293] In search of the markers for piperaquine resistance, two studies were further done on patients from areas where DP is failing. Amato *et al.*[294] used a genome-wide association study (GWAS) platform with increased piperaquine IC_{50} as the basis[294], while Witkowski *et al.*[295] used PSA survival rates and whole-genome sequencing to identify single-nucleotide polymorphisms (SNPs) and copy number variations. GWAS identified *exo*-E415G in chromosome 13 as the best candidate for increased piperaquine IC_{50}[294], and amplification of *plasmepsin* 2 and *plasmepsin* 3 copy numbers in chromosome 14 was strongly correlated with increased piperaquine IC_{50}[294] and PSA survival rates[295]. In addition, proportion of *exo*-E415G[294] and multicopy *plasmepsin* 2-3 in those population increased over time. Further to validate these novel markers, both studies investigated the association between the markers and DP treatment outcomes. Recrudescent patients were associated with increased piperaquine IC_{50}, PSA survival rates and DP failures. Interestingly, Amato found single copy *pfmdr1* was equally distributed in recrudescent and non-recrudescent samples[294], while Witkowski showed a higher risk to fail treatment if harboring a single copy *mdr1* (RR 3.0 [95% CI 1.4-6.3], p=0.003)[295]. Multicopy *plasmepsin* 2 was a strong predictor for DP failures[294], with copy numbers of three or more are more frequent in recrudescent patients (Figure 1.18.)[295]. Analysis on archived samples revealed amplified *plasmepsin* 2 gene has occurred in western Cambodia since 2008 and later in Thailand in 2014-2015 with no amplification in isolates from Laos and Myanmar. The presence of multicopy *plasmepsin* 2 only in the back of artemisinin-resistant parasite (harbouring *pfk13* C580Y) implied that this amplification occurred after the selection of artemisinin-resistant genotypes.[296]

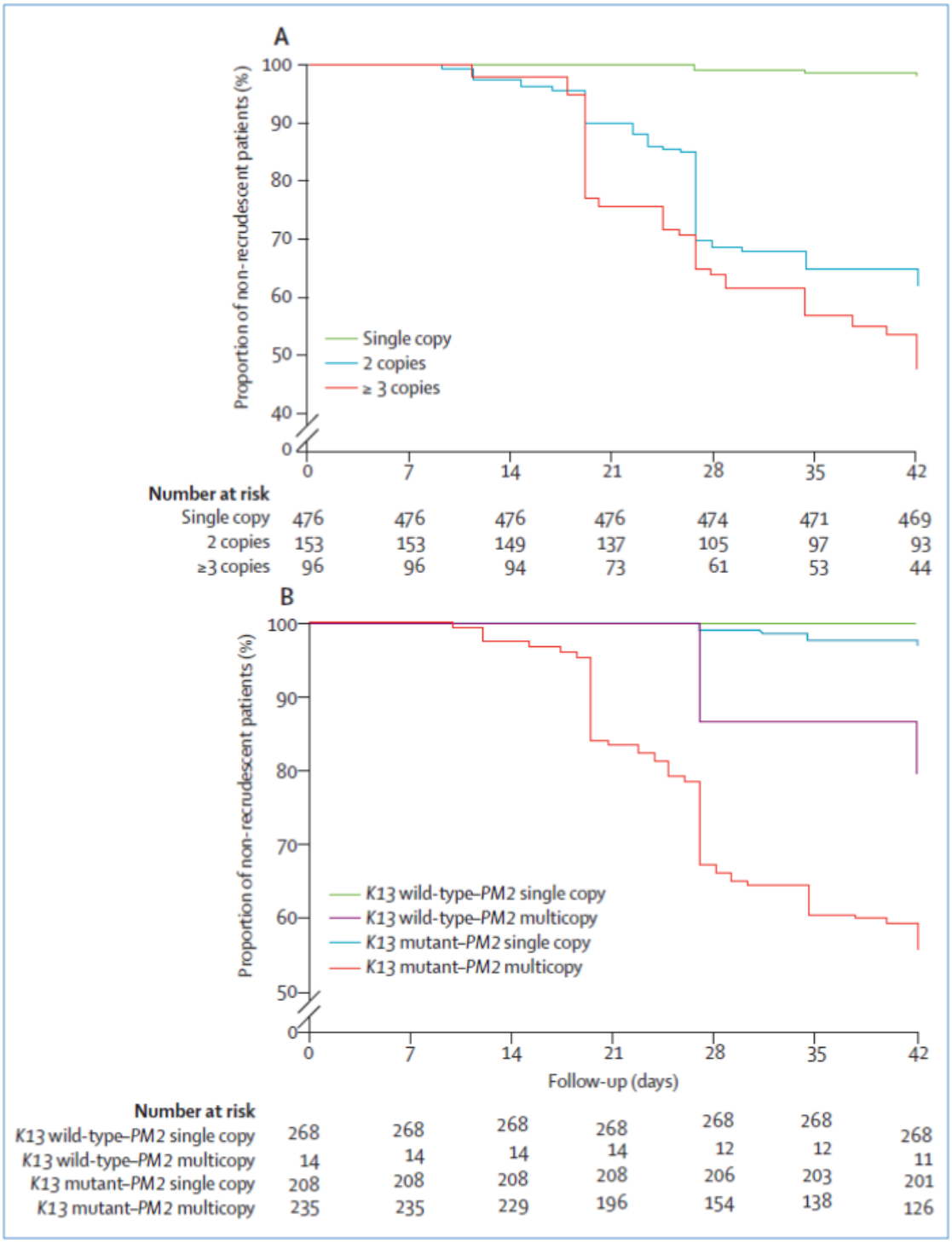


Figure 1.18. Cumulative proportion of non-recrudescent patients treated with full-course DP. (A) *Pfplasmepsin2* gene copy number at enrollment before treatment; (B) *Pfplasmepsin2* copy number and K13 genotype at enrollment before treatment. Adapted from reference [295].

1.8.1 Molecular marker for artemisinin

There have been several candidates for molecular markers for *P. falciparum* resistance to artemisinin including *pfatp6*, *G7*, *pfmrp1*, *pfubp1*, and *pfap2-mu*. However, there is not enough evidence yet to link the mutations in these genes with clinical artemisinin resistance.[170] In ACT-treated children in Kenya, persistent submicroscopic infections were present on day 3 and polymorphisms in *pfap2-mu* S160N/T and *Pfubp1* E1528D were significantly higher than pre-treatment. However, the variant genotypes in both genes are not associated with Southeast Asian resistant-parasites where reduced efficacy of artemisinin associated to slow-clearance has been established.[297]

One study performed a targeted association analysis to identify the genome region underlying artemisinin resistance using 91 isolates from Cambodia, Thailand and Laos. This study identified 33 genome regions, 10 of which accounted for previously known antimalarial resistance genes. Chromosome 13 showed a strong association with slow clearance rates, and several candidate genes were selected from that region.[298] Further work to discover molecular markers for artemisinin resistance used laboratory-adapted parasite clones that survive high doses of artemisinin *in vitro*.[268] Mutations acquired in this parasite were analysed and used as a guide to analyse polymorphisms in clinical parasite isolates from Cambodia. While no mutations at *pfcrt*, *pftctp*, *pfmdr1*, *pfmrp1*, ABC transporters, *pfatp6* and *pfubp1* were seen, mutations at K13-propeller domain were identified. Four-mutant alleles (Y493H, R539T, I543T or C580Y) were associated with parasite phenotypes determined by the ring-stage assay$_{0\text{-}3\ h}$ ($RSA_{0\text{-}3\ h}$) survival rates. Subsequently, K13 polymorphisms analysis was performed on *in vivo* study on artemisinin efficacy. Forty-two percents (72/150) harbored wild-type allele and the remaining (58%) carried single non-synonymous SNP in the *pfk13*-propeller: C580Y, R539T, or Y493H. Patients with any of the three K13 propeller mutant-alleles showed significantly slower clearance.[268] Further Straimer *et al.* studied whether K13-propeller mutations mediate artemisinin resistance and explored the contribution of the mutations to the levels of resistance by genetic modification using zinc-finger nucleases (ZFNs) method. Removal of K13 mutations from culture-adapted Cambodian isolates showed to reverse the response in the $RSA_{0\text{-}3h}$ survival rates, while introduction of K13 mutations into Dd2 parasites correspond to increased $RSA_{0\text{-}3h}$ in varying level depending on which point mutation was present (Y493H < C580Y < M476I < R539T < I 543T). Thus, these findings suggested K13 mutations confer different levels of resistance to artemisinin and strengthened the importance to track the presence of K13 mutations in a large-scale surveillance.[299]

To further investigate the association of this resistance marker with phenotypic profile of slow clearing infections, randomised controlled trials on the efficacy of ACTs were done involving 10 countries (Cambodia, Thailand Laso, Vietnam, Myanmar, Bangladesh, India, Nigeria, Kenya, and the Democratic Republic of Congo). Prolonged parasite clearance (parasite clearance half-life > 5 hours[vi])[300] were predominantly observed in patients from Cambodia and Thailand (49 to 73%), with a higher chance to occur in the presence of point mutation after position 440 in the propeller domain of K13 (OR 94.7, 95% CI 54.6 to 164.0, *P*<0.001) (Figure 1.19.).[255] Mutations in *pfk13* were shown to be determinants of the half-life of parasite clearance.[255, 301]

[vi] Parasite clearance half-life is estimated by measuring parasite density from serial microscopic parasite count data to assess to which extent ring-stage parasites are killed and removed from the circulation, with time relationship between log-transformed parasite density and time generally linear. A tool to measure this rate is developed by WorldWide Antimalarial Resistance Network.

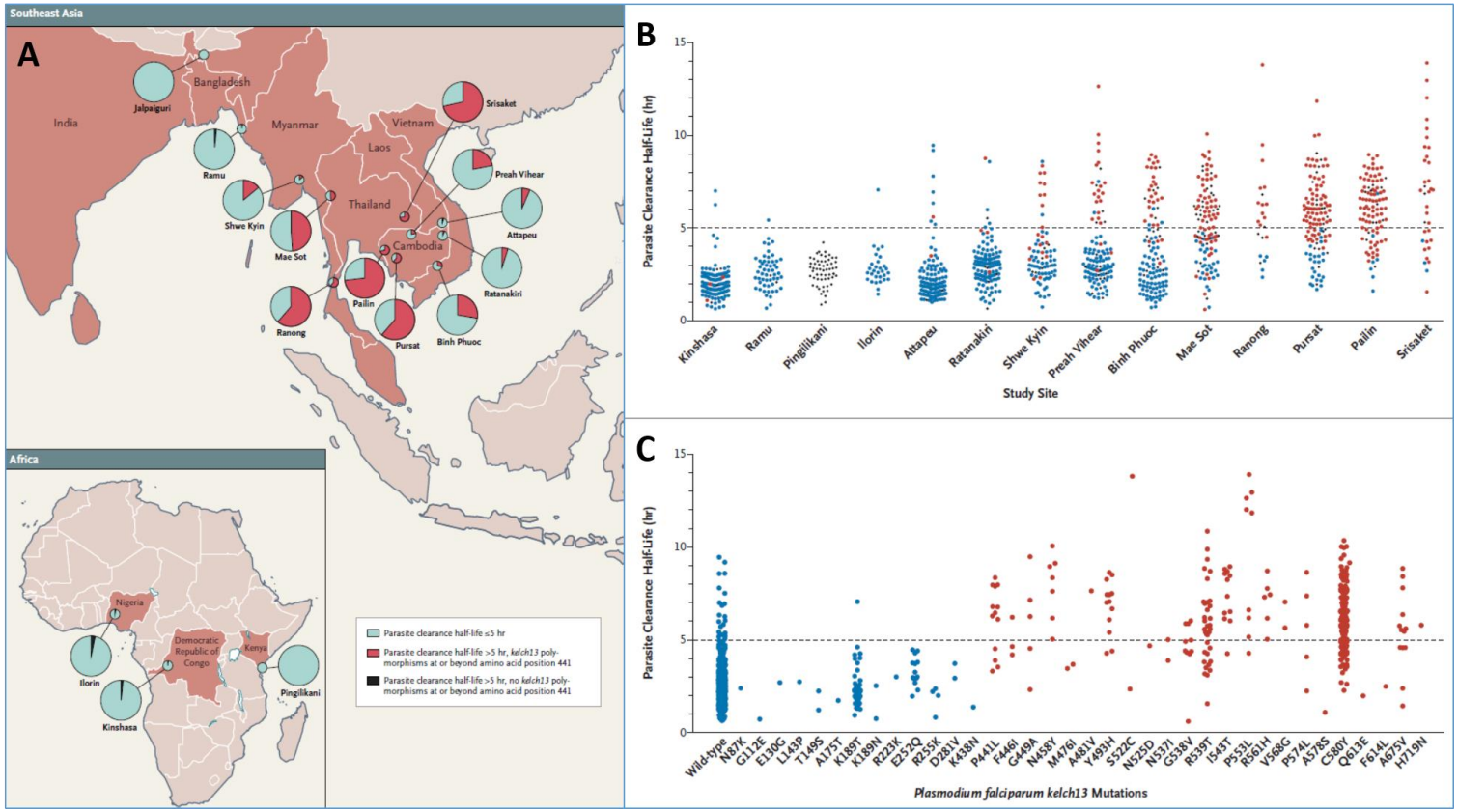


Figure 1.19. (A) Proportion of patients with clinical and K13 profiles according to study sites; (B) Parasites clearance half-lives according to study sites; (C) Polymorphisms in the propeller domain of K13 gene in relation to the parasites clearance half-life. One circle represent one pasient. Polymorphisms in the *pfk13* are indicated by blue circles (wild-type or mutation before position 441), red circles (mutation after position 440), or black circles (at least part of K13 sequence is missing or heterozygous). Adapted from reference [255].

Increasing concerns that artemisinin-resistant parasites might disseminate from its epicenter in Cambodia have led to the efforts to map the geographical extent of the *pfk13* mutants. Early detection of mutations will allow understanding whether resistance have spread or emerged independently and to implement strategies to contain the parasites. Menard *et al.* sequenced 14,037 samples from 59 countries to identify the *pfk13* polymorphisms linked to the geographical origins. 108 nonsynonymous K13 mutations were identified with 9 had frequency > 1%; C580Y, F446I, R539T, A578S, Y493H, P574L, P553L, N458Y, and R561H (Table 1.5.).[302] Most mutations were distributed in two regions in Southeast Asia exhibiting distinctive profiles: Cambodia, Vietnam, Laos were prevalent for C580Y, R539T, Y493H, I543T; while Thailand, Myanmar, China were prevalent for F446I, N458Y, P574L, R561H (Figure 1.20.).[296, 302, 303] Three mutations (493H, 574L, and 580Y) occurred in both populations. However, subsequent haplotype analysis based on linkage disequilibrium in flanking areas of K13 suggested 493H to originate from Cambodia and spread to Vietnam, and 574H and 580Y arose focally in Vietnam or Vietnam/Cambodia and Myanmar (Figure 1.21.). There was no evidence that resistant-parasites have spread from Cambodia to Myanmar, and K13 mutations emerged independently in the two regions.[301, 304] In Africa, K13 mutations have also been documented, however different to those (580Y, 539T, 493H) associated with artemisinin resistance in Southeast Asia. Novel mutations have been observed with mutation at A578S appeared in several countries with yet unknown phenotypic impact.[305-307] In high transmission settings, patients' immunity usually clears parasite residuals, therefore measuring the parasite clearance times is probably not the best indicator to detect resistance in this region. A case report of patient from Equatorial Guinea with day-3 positivity after full course of DP identified mutation at M579I in the propeller domain of K13.[308] However, this single case is not sufficient to correlate K13-propeller mutations with artemisinin resistance in Africa. Limited evidence on the association of clinical resistance with K13 mutations in African isolates, suggesting there is a different mechanism mediating artemisinin resistance in this region.[305, 306, 309]

Table 1.5. List of K13 point mutations and their association with clinical resistance and validation by *in vivo* and *in vitro* data. Adapted from reference [203].

K13 mutation	Classification
E252Q	Not associated
P441L	Candidate
F446I	Candidate
G449A	Candidate
N458Y	Validated
Y493H	Validated
G538H	Candidate
R539T	Validated
I543T	Validated
P553L	Candidate
R561H	Validated
V568G	Candidate
P574L	Candidate
A578S	Not associated
C580Y	Validated
A675V	Candidate

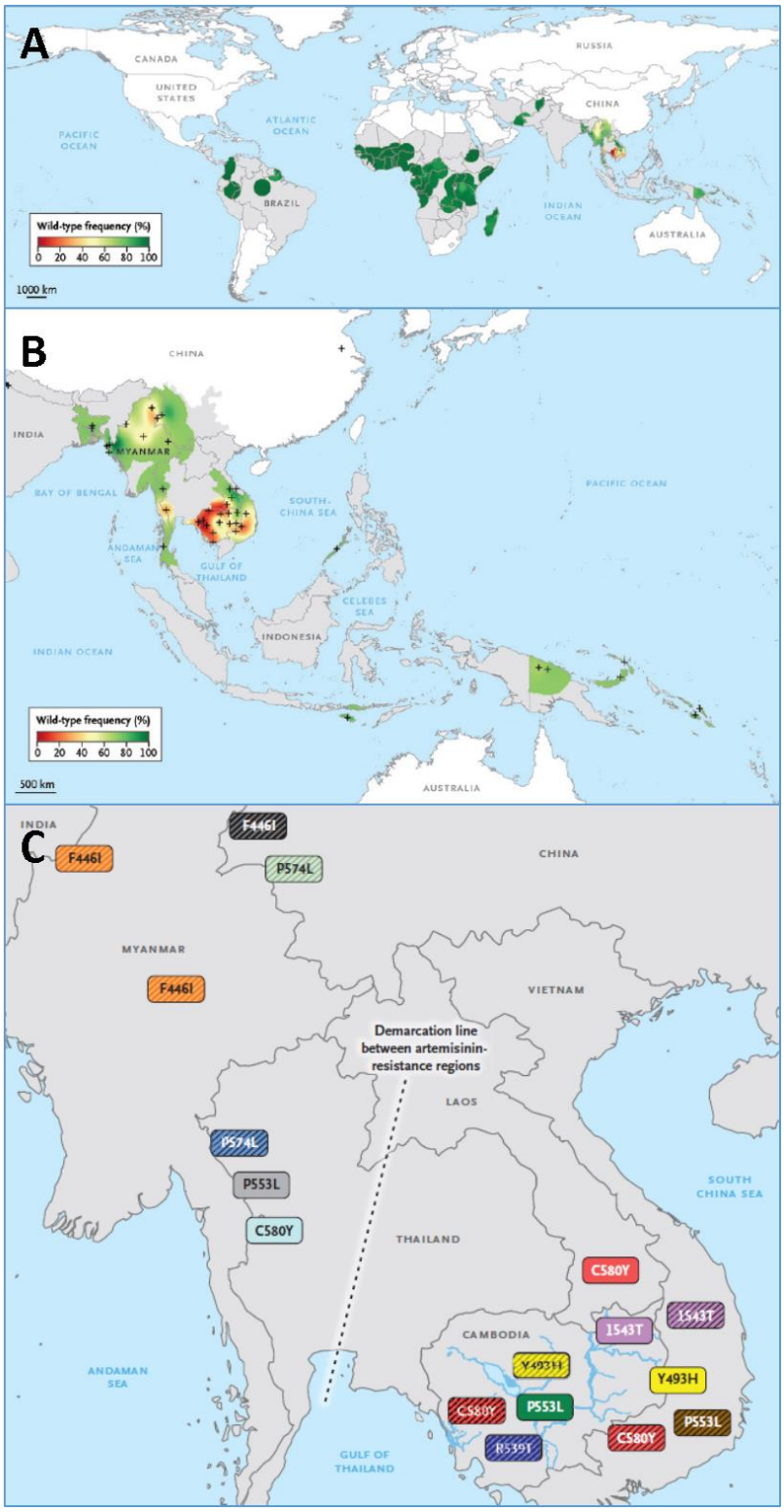


Figure 1.20. (A) Frequency distribution of the wild-type *pfk13* allele worldwide; (B) Frequency distribution of the wild-type *pfk13* allele in Asia; (C) Distribution of the common mutant alleles of *pfk13* in Southeast Asia. Colored boxes and hatches boxes correspond to shared haplotypes and the presence of additional country-specific haplotypes, respectively. Adapted from reference [302].

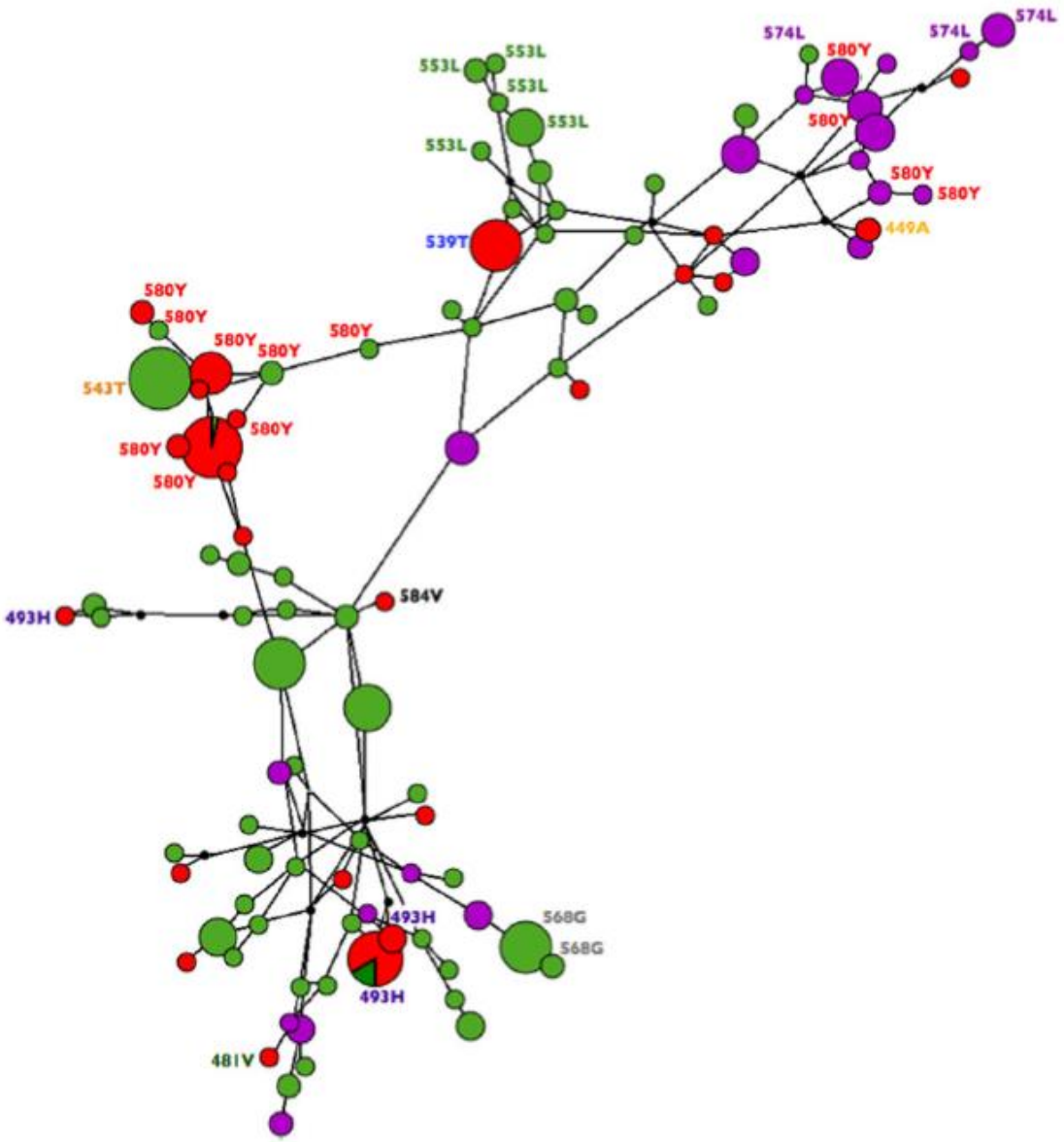


Figure 1.21. *P. falciparum* K13 mutations within linkage disequilibrium of the K13 gene. Prevalence of haplotype is proportional to size of the circle. Red color indicate Cambodia origin, purple color indicate Myanmar origin, green color indicate Vietnam origin. Adapted from reference [301].

1.9 Actions to ACTs resistance

Declining efficacy of ACTs in Southeast Asia is highly concerning and a real threat for malaria-endemic countries. WHO launched a Global Plan on Artemisinin Resistance Containment in 2013 with detailed actions to contain resistant parasites from spreading to high endemic regions. Nevertheless, K13-propeller mutations associated with artemisinin resistance have emerged independently in at least two sites in the GMS, and resistance to partner drug (e.g. piperaquine) may also have emerged independently. Efforts to control malaria and to achieve elimination require new strategies to prevent increased morbidity and mortality. Several proposed strategies including:[310, 311]

- Extending current ACT course from 3 days to either 5 or 7 days
- Re-ACT or administration of two different 3-day ACT in sequence
- Rotating two ACTs
- Deployment of multiple first-line therapies
- Triple ACTs (combination of artemisinin with two partner drugs)

As Indonesia progresses towards elimination, the threat of spread or emergence of *P. falciparum* resistance to current recommended treatment into this corner of Southeast Asia is alarming. There is a need to establish a strong surveillance system to evaluate the progress made to achieve elimination, to monitor the efficacy of the recommended ACT in this region, thus treatment failures due to drug resistance can be avoided and malaria elimination goal can be achieved.

Chapter 2

AIMS & OBJECTIVES

2 AIMS

The work described in this thesis aims to improve our understanding on malaria distribution in North Sumatera, Indonesia during the malaria elimination era and to investigate the efficacy of current ACTs where emergence of multi-drug resistance parasites is prevalent in the neighboring countries. The research has two major aims described below.

Aim 1. To investigate malaria distribution in a low transmission setting in North Sumatera province, Indonesia.

Specific objectives:

1. To identify the presence of all *Plasmodium* species and to determine the proportion of each species
2. To determine the contribution of subpatent infections to the overall malaria burden
3. To provide genetic profiles associated with polymorphisms in drug resistance markers to current recommended antimalarial treatment among *P. falciparum* infections

These objectives are addressed by work described in chapters 4 and 5.

Aim 2. To evaluate the efficacy of dihydroartemisinin-piperaquine and artemether-lumefantrine for the treatment of uncomplicated *P. falciparum* malaria and as post-treatment prophylaxis.

Specific objectives:

1. To determine the proportion of treated patients showing adequate clinical and parasitological response (ACPR) at day 42, for each of the two ACTs
2. To measure the *P. falciparum* clearance dynamics in the 72 hours after treatment
3. To investigate the association between genetic polymorphisms and treatment outcomes , using the established WHO definition of ACPR

4. To determine the proportion of subpatent recurrent infection at day 28 and day 42 after treatment using molecular assays, and relate this to genetic variation in parasite genes implicated in ACT susceptibility

These objectives are addressed by work described in chapters 6 and 7.

Chapter 3

MATERIALS & METHODS

3 MATERIALS AND METHODS

3.1 Study design

This study consisted of two study designs. The first study is a parasitological survey in three study sites in North Sumatera province, Indonesia. The second study is a prospective, open label, randomised controlled trial to assess the safety and efficacy of dihydroartemisinin-piperaquine (DP) and artemether-lumefantrine (AL) in children and adults with uncomplicated *P. falciparum* malaria infection.

3.2 Study sites

The study was conducted in three regencies in North Sumatera province, Indonesia. North Sumatera province is located at 2°00'N 99°00'E (Figure 3.1.) with a total land area of 71,680.68 km^2. The total population in 2014 was 13,766,851 with equal ratio between male and female. There are 8 cities and 25 regencies, with Medan as the capital city. Productive age (15-64 years of age) accounted for the most proportion in this population.[312]

There are two seasons occurring in this area with a transition period in between. Dry season starts from June to September and rainy season begins in November to March. The temperature ranges from 13.4° C to 33.9°C.[312] The transmission intensity for *P. falciparum* infection is stable in approximately half of the region but stands at low risk. In 2010, Elyazar *et al.* estimated a quarter of population in this region were exposed to *P. falciparum* infection.[88]. A total of 44,403 malaria cases was suspected in 2015, and 99% underwent diagnostic tests by microscopy or rapid diagnostic test resulted in 3,503 positive cases. From 2008 to 2013, there were an increasing trends in annual parasite incidence before declining in the following year (Figure 3.2.).[93, 313]

Figure 3.1. Map of North Sumatera province, Indonesia with the three studied regencies indicated. Adapted from reference [29].

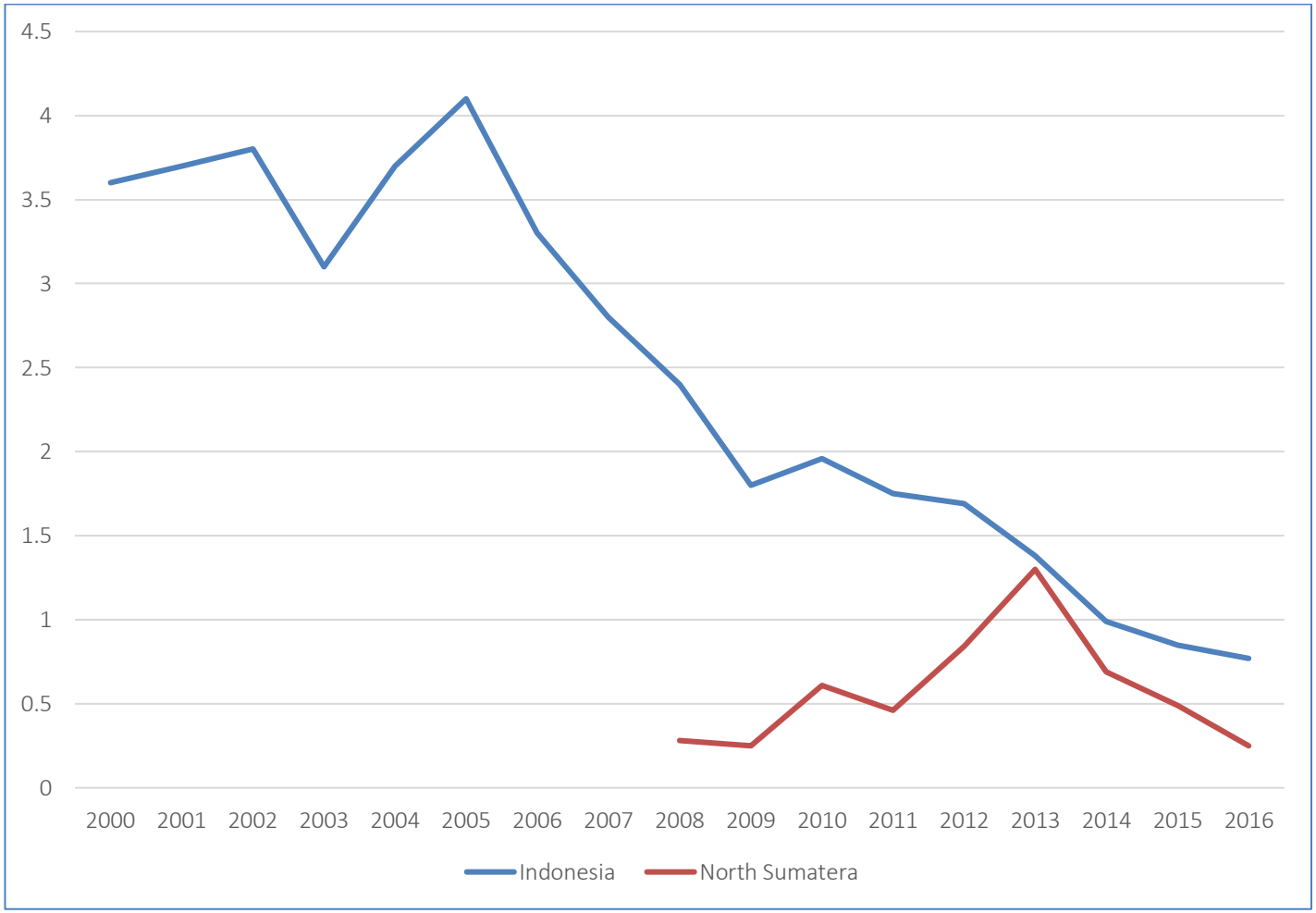


Figure 3.2. Annual parasite incidence in North Sumatera province and Indonesia, 2000 - 2016. Based on data from reference [87, 93, 314].

Three study sites: Batubara, Langkat and South Nias regencies were selected based on published malaria endemicity.[315] Batubara is located on the east coast of Sumatera facing peninsular Malaysia with a size of 904.96 km^2. It comprises semiforested and plantation areas, with 37.31% of productive age population was working in agriculture, plantation, forestry or fishery. There were 2 public hospitals, 14 primary health centres and 60 health posts in addition to 1 private hospital and 127 private clinics/pharmacies to cover the 396,479 population. Primary health centre runs 6 days a week from 8 am to noon. We also established a 24-hour 7-day clinic to capture more patients with fever. We conducted health promotion and education on malaria at 47 primary health centres, health posts, schools and head village offices (Figure 4.1.) during 8-week period of screening. Primary health centres also referred patients with positive slide or RDT to our clinic. Bahasa Indonesia and/or local dialect Malay with the help of a translator were used to communicate with patients. There had been a significant increase of annual malaria incidence (AMI) in this regency between 2010 and 2014 from 8.52 to 24.34 per 1,000 population due to increased funding for malaria diagnostic tests.[316]

Langkat regency is a forested highland area with a size of 6,263.29 km^2 located 105-530 m above sea level. There were 967,535 population in Langkat with over 50% of population over 15 years of age was also working in agriculture, plantation, forestry or fishery. There were 1 public hospital, 30 primary health centres and 171 health posts supported by 5 private hospital and 127 private clinics. Nevertheless, villages we visited in this regency were isolated with very poor access to health facilities (Figure 4.2.). We visited 10 villages and malaria screening by active case detection was done for 2-3 days in each village.[312, 317] In this study site, Bahasa Indonesia and/or local dialect Karo with the help of a translator were used to communicate with patients.

South Nias regency is cluster of islands in Indian ocean. Main part of the regency is situated in Nias island, with the rest of the region scattered in 101 small islands named Batu islands (islands of rocks). There were 289,708 population in 1,625.91 km^2 of its total area. We set up a-24 hour 7-day clinic in Tello island which functioned also as a diagnostic laboratory. We visited 32 villages and schools spread across 7 islands: Tello, Tanahmasa, Balogia, Sipika, Pono, Sibaranun, and Marit (Figure 4.3.), however most villages were not connected by land. Malaria screening involved daily sea journeys to conduct malaria sensitization in the community, followed-up by screening of individuals with fever and healthy individuals who

requested for a malaria test.[312] The local dialect Nias was used to communicate with patients in this study site.

3.3 Study duration

Samples were collected between January and June 2015.

3.4 Sampling

We performed passive and reactive case detections at each regency. Passive case detections were done for groups of patients who visited primary health centres or our 24-hour clinic with fever (axillary temperature $\geq$ 37.5 °C) or a history of fever in the preceding 48 hours. Reactive case detections were conducted on household members and neighbours of index case detected during passive surveillance. Individuals who appeared with fever or history of fever, and healthy volunteers during community/school sensitization were also tested for malaria as part of active case detection.

3.5 Enrollment to drug efficacy study

Patients aged > 6 months of age with fever or history of fever in the preceding 48 hours and confirmed by microscopy for uncomplicated *P. falciparum* infection.

3.5.1 Sample size calculation

In vivo therapeutic efficacy studies on DP in Indonesia showed high efficacies at day 42.[176, 193, 209] We used the efficacy result from previous study in South Sumatera with 100% ACPR.[209] With the estimation of the efficacy for artemether-lumefantrine of 95%, we calculated the sample size for comparing two proportions.[318]

$$n1 = n2 = f(\alpha, P)\ \frac{(p1\ (1 - p1) + p2\ (1 - p2))}{(p1 - p2)^2}$$

n1 = n2	:	number of subjects in one group
α	:	statistical significance
P	:	power
*p*1	:	Proportion of subjects with ACPR in group 1
*p*2	:	Proportion of subjects with ACPR in group 2

Based on the calculation above, using a power of 80% and a significance level of 0.05, a total of 300 subjects in the study will provide us with a statistical significant difference in treatment outcomes between dihydroartemisinin-piperaquine and artemether-lumefantrine.

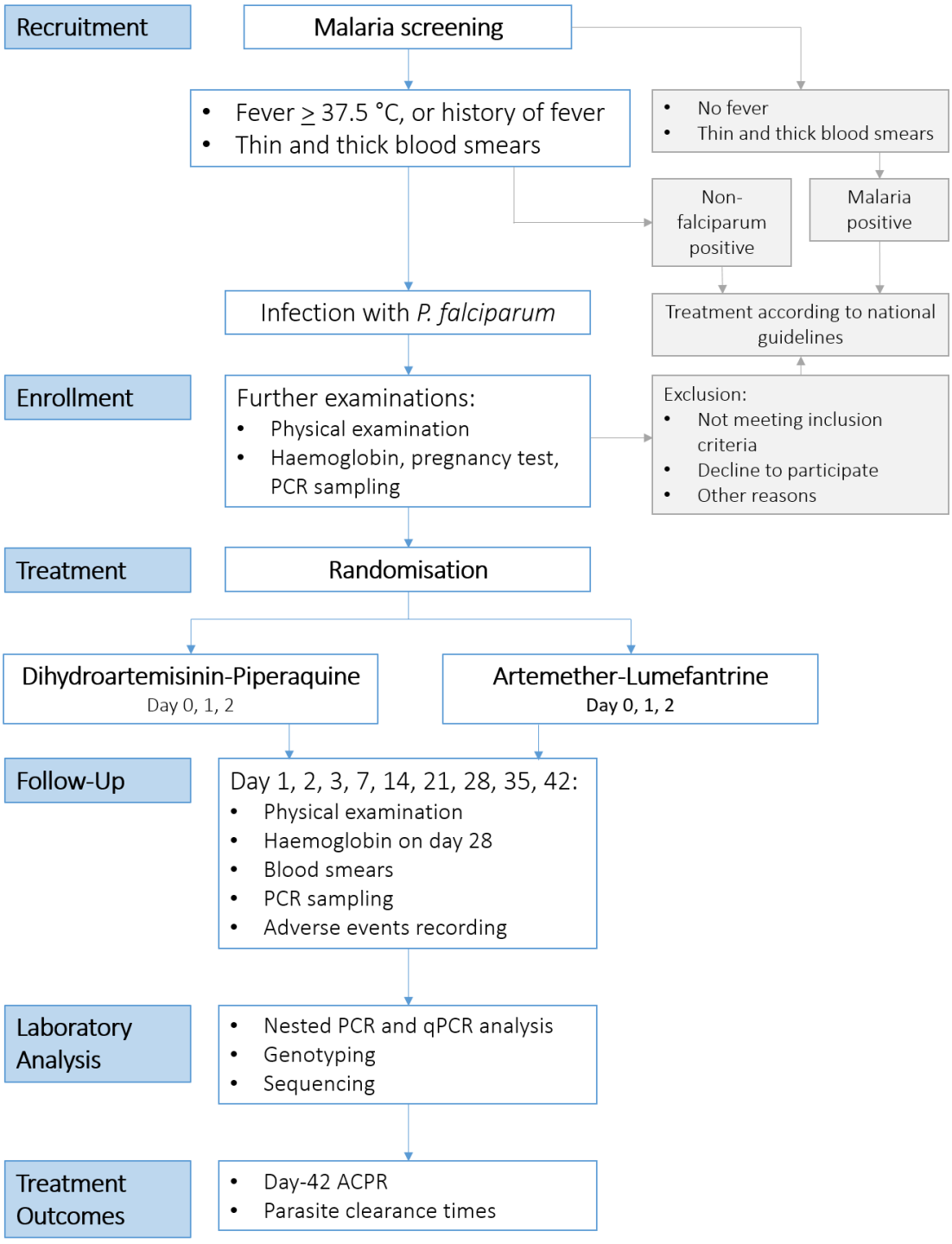
3.6 Ethical clearance

The study was approved by the ethic committees of the London School of Hygiene and Tropical Medicine, United Kingdom (identifier 8504-01, Appendix 1) and the University of Sumatera Utara, Indonesia (identifier 401/KOMET/FK USU/2014, Appendix 2).

3.7 Randomisation

Randomisation list was generated using excel software. Pre-assigned treatment was printed on numbered case record form for 300 participants.

3.8 Study protocol flow diagram



3.9 Study procedures

Table 3.1. describes lists of assessments conducted during the study, all information was recorded in the case screening forms (Appendix 3) and case record forms (CRF, Appendix 4)).

Table 3.1. List of evaluation tasks during study period

Evaluation	Enrollment	Treatment							Follow-Up					
Days	0			1		2		3	7	14	21	28	35	42
Hours		0	8	24	36	48	60	72						
Demographic data														
Medical history														
Concomittant treatment														
Vital signs and body weight														
Physical examination														
Blood smear														
Gametocyte count														
Rapid diagnostic test														
Hematology and biochemistry tests														
Pregnancy test														
Drug administration														
PCR sampling														
Adverse events														

3.10 Recruitment

During the recruitment period, the following activities were performed including basic screening, evaluation for enrollment, and informed consent.

3.10.1 Basic screening

We performed blood smears, rapid diagnostic tests (RDTs), when available, and filter paper blood spots sampling on patients presenting at the study clinic with fever (axillary temperature > 37.5 °C) or history of fever in preceding 48 hours, and also on healthy individuals who volunteered to have malaria tests. Baseline information including age, sex, body weight and height were recorded as part of the initial screening. Giemsa stained thick (and thin) blood films were prepared to screen asexual parasites and gametocytes at 1000x magnification microscopy examination. Thin films were used to identify *Plasmodium* species. Thick films were used to determine parasite density by counting the number of asexual forms of *P. falciparum* parasites per 200 WBCs, with an assumption that white blood cell density is 8000 per μL.

$$\text{Parasite density / } \mu\text{L} = \frac{\text{Number of parasites counted x 8000}}{\text{Number of leukocytes counted (200)}}$$

A blood smear was considered to be negative after examination in 100 microscopic fields. To detect sexual forms of parasite, the presence of gametocytes was screened against 1000 leukocytes. All information was recorded in the case screening form (Appendix 4). Repeated blood films were done for enrolled participants as scheduled above and until the parasite was cleared from the slides.

In addition to microscopy examination, we also performed RDT as a comparison. RDTs examination is the standard diagnosis for malaria in the local health clinics especially when trained microscopist is not available. CareStart™ Malaria HRP2/pLDH (Pf/Pan) Combo, SD Bioline Malaria Ag Pf/Pan, Parascreen®-Rapid Test for Malaria Pan/Pf, FalciVax™-Rapid Test for Malaria Pv/Pf, and Paracheck® Pf-Rapid Test for *P. falciparum* malaria device were provided by the Provincial Department of Health and the LSHTM, and it was used based on

availability. For this procedure, during the finger prick blood sampling for blood films preparation, a full drop of blood (±5 μL) was taken using the loop supplied by the manufacturer RDT package and placed onto the filter paper in the RDT device. Six drops of buffer were added to the well. Results were interpreted after 15 minutes and positive result was confirmed by identifying two lines (control and test) on the device. The microscopists were blinded from the RDT results. Patients must have positive blood slides to be enrolled to the study. Patients with positive RDT results but negative blood slides were treated for malaria but were not eligible for the study.

Three drops of blood were spotted onto 3MM Whatman filter paper to be examined by nested PCR at LSHTM for quality control. The filter papers were left to dry overnight and attached to a labeled cardboard cover on the following day. A set of 30-40 covers with attached filter papers were kept in a plastic bag containing silica gel. Hemoglobin level was determined using HemoCue® to exclude severe anemia.

3.10.2 Evaluation for enrollment

Study doctors evaluated patients who fulfilled the basic criteria for enrollment. Participants were assessed for inclusion and exclusion criteria. A case screening form (Appendix 3) was used to document participant's information. Doctors carefully evaluated the presence of danger signs, severe malaria, severe anemia and also other febrile causes to exclude participants from the study.

3.10.2.1 Inclusion criteria

- Male or female;
- All patients over 6 months of age;
- Fever as defined by axillary temperature > 37.5 °C or history of fever during the 48 hours before recruitment;
- Infection with *P. falciparum* detected by microscopy;
- Parasitaemia >250 /μl blood;
- Ability to swallow oral medication;
- Ability and willingness to comply with the protocol for the duration of the study and to comply with the study visit schedule;

- Informed consent from the patient of from a parent or guardian in the case of children;
- Absence of history to hypersensitivity reactions or contraindication to antimalarial drugs;
- Not currently consuming antibiotic with antimalarial activity (such as cotrimoxazole, macrolides, tetracycline or doxycycline) (Appendix 5)

3.10.2.2 Exclusion criteria

- Presence of *P. vivax* infection detected by microscopy;
- Presence of general danger signs in children under 5 years or signs of severe falciparum malaria according to the definitions of WHO (2000) (Appendix 6);
- Presence of severe malnutrition according to WHO child growth standards (Appendix 7);
- Presence of febrile conditions caused by diseases other than malaria;
- Presence of severe anemia (Hemoglobin < 7 gr/dl);
- Received any of the study drugs within the past 4 weeks;
- Received any antimalarial within the last 2 weeks;
- Recurrent vomiting (necessitating more than a single repeat dose);
- Pregnant (demonstrated by positive result of β-HCG in women of childbearing age);
- Lactating mother

3.10.2.3 Criteria for withdrawal from the study

- Withdrawal of consent;
- Persistent vomiting;
- In need of parenteral treatment;
- Severe adverse events related to study drug;
- Hypersensitivity or allergy to study drug;
- Received any antimalarial or antibiotic with antimalarial activity during study period;
- Move out from study area;

- Failure to complete the treatment

3.10.2.4 Amendments to the protocol

After the protocol has been accepted, amendment on exclusion criteria was made with agreement of the principal investigator and the LSHTM and University of Sumatera Utara ethic committees. Amendments were made in the exclusion criteria where the first point of the criteria "Presence of *P. vivax* infection detected by microscopy" was omitted.

3.10.3 Informed consent

Participants received full information about the study including the purpose of the study, procedures, compensations, disadvantages or risks from taking part, benefits, confidentiality, discontinuation, and duration of study according to the rules of international and local guidelines for research relating human subjects (Appendix 11 and Appendix 13). Information was given in either Bahasa Indonesia or local dialect Malay, Karo or Nias translated by a local translator. Participants were given time to ask questions and to consider whether they agree to be enrolled to the study. Signed informed consents (Appendix 12 and Appendix 14) were sought from participants or guardians of children (age < 15 years).

3.11 Enrollment

Participants who met all selection criteria and signed gave written informed consent were further interviewed for demography and medical history recording, and also had physical and laboratory examinations.

3.11.1 Demography and medical history recording

Participant's name, address, occupation as well as medical history were recorded. The address was particularly important to reach patients at their home when they failed to appear on follow-up days. Patients' identification (name and address) was not entered to digital documents.

3.11.2 Physical examinations

Physical examination was done thoroughly at every scheduled visit. Physical examinations comprised examinations on general appearance, skin, ear, throat, respiratory, cardiology, abdominal, genito-urinary and extremities. All abnormalities were documented in the CRF (Appendix 8) and the examiners assessed the need for any concomitant medications.

3.11.3 Laboratory examinations

- Hemoglobin level was determined using HemoCue® at enrollment and day 28.
- Pregnancy test from urine was done for childbearing age women at initial screening.
- Thin and thick films were examined on day 1, 2, and 3 or until patients cleared the parasites, as well as at the following scheduled visits (day 7, 14, 21, 28, 35 and 42) or at any day patients showed up at the clinic, and blood film examination was requested by the study staff.
- Filter paper blood sampling was performed for *post hoc* PCR analysis to determine *Plasmodium* species, to measure parasite clearance times and to detect molecular markers.

3.12 Treatment

3.12.1 Treatment

- Treatment with dihydroartemisinin-piperaquine (Duo-Cotecxin, Zhejian Holley Nanhu, Guangzhou, China)

  One tablet of DP consists of 40 mg of dihydroartemisinin and 320 mg of piperaquine. DP was administered once daily for 3 days (at enrollment, hour 24, and hour 48). Dosing was given based on body weight. Daily dose for dihydroartemisinin is 2.25 mg/kg (total 6.75 mg/kg) and for piperaquine is 18 mg/kg (total 54 mg/kg). DP is the first line treatment in Indonesia.

- Treatment with artemether-lumefantrine (Coartem, Beijing Novartis Pharma Ltd, Beijing, China)

  AL was administered as 6-dose regimens given twice daily for 3 days (at enrollment, hour 8, hour 24, hour 36, hour 48, and hour 60). Half a tablet consisting of 20 mg of artemether and 120 mg of lumefantrine was given per 5 kg body weight.

3.12.2 Treatment administration

Administration of the drugs for DP was supervised at the study center or during home visit at enrollment, day 1 and day 2. Administration of the first dose of AL for each day was given at the study center and for the second daily dose, study staff visited the patient at home if feasible to observe drug administration. Subjects in DP group were visited in the afternoon to ensure equal monitoring between two groups. AL was given with drinking water on a full stomach, and food was provided for participants with an empty stomach. While DP was given in an empty stomach, patients were instructed to fast for 2 hours after drug administration. For paediatric participants, the tablet was crushed to meet the correct dosage and then dissolved in drinking water in a small cup. After drinking the water, 10 ml of drinking water was added to the cup to be administered to the patients again. Hereafter, study staff observed participants for 1 hour after drugs administration, if a participant vomited within 30 minutes then a full dose was repeated and if between 30 minutes and 1 hour a half dose was given. Recurrent vomiting would withdraw participants from the study and receive rescue medication.

Antimalarial and other medicines were provided to participants, non-recruited patients and withdrawn patients. Medicines for participants are listed as non-allowed concomitant medicines, allowed concomitant medicines and rescue medicine. Non-allowed concomitant treatment includes any antimalarial drugs and antibiotic with antimalarial action such as cotrimoxazole, macrolides, tetracycline or doxycycline. Allowed concomitant medicines are anthelminthics, analgesics, other antibiotics without antimalarial action such as penicillins, cephalosporins, hematinics. The use of antipyretic was allowed (if necessary). Any concomitant medicine was recorded in the CRF (Appendix 4). Rescue medication was given to participants who develop signs of treatment failure within 42 days according to WHO definitions (Table 3.2.). Participants were withdrawn from the study and received second-line

treatment according to the national treatment protocol. Current recommendation is quinine (10 mg of salt/kg three times a day for 7 days), with additional doxycycline (100 mg twice a day for 7 days) for children > 8 years of age and non-pregnant women. Non-recruited participants received treatment according to the standard of care in the national guidelines.

3.13 Follow-up

Follow-up schedule comprised of visits on day 3, day 7 and then weekly after day 7. However, participants were also asked to return on any day the symptoms reappear. Follow-up observations included assessment on safety and tolerability of treatment throughout the study. Safety evaluation consisted of monitoring of adverse events, measurement of vital sign and body weight, physical examination, hemoglobin evaluation, and thin and thick blood films to verify presence of parasites. Parasite density were measured at each reading. Presence of gametocytes was recorded on follow up days 3, 7, 14, 21, 28, 35 and 42. Microscopists remained unaware of treatment allocation until the end of the study. Filter paper blood sampling was done at each visit as scheduled and also at any day participants felt unwell. All results were recorded on the CRF (Appendix 4) and adverse events form (Appendix 9).

Adverse events were assessed by direct interview. Adverse events were defined as a condition of any unfavourable, unintended sign, symptom, syndrome or disease that develops or worsens concomitant with the use of study medicines, regardless of whether it is related to the study medicines. Occurrence of an adverse event was classified based on the severity, its relationship with the study drugs, duration of adverse events, which actions taken, the outcomes, and the person who has identified the adverse event according to established clinical trial practise (Appendix 9).[172]

A serious adverse event is defined as any untoward medical occurrence that at any dose results in death, is life threatening; requires hospitalization or prolongation of hospitalization; results in persistent or significant disability or incapacity; or is a congenital anomaly or birth defect; or is medically significant that jeopardizes the participant or requires intervention to prevent one of the previously mentioned outcomes. If a serious adverse event was identified, a report was made immediately within 24 hours to trial monitor (Appendix 10).[172]

3.14 Laboratory analysis

All samples collected at enrollment were screened for any *Plasmodium* infection for confirmation using nested PCR following the protocol by Snounou *et al*.[319] Extraction of parasite DNA from filter paper was performed using the Chelex-based method.[320] Parasite clearance times for enrolled patients were determined using qPCR.[321]

Polymorphisms in genes associated with drug resistance markers for antimalarial used in the study including *pfcrt* gene, *pfmdr1* gene and *pfk13*, encoding the kelch13 propeller domain protein, were determined using published methods.[270, 273, 322] Samples from day of treatment failure were investigated by *msp1* (merozoite specific protein), *msp2* and *glurp* (glutamate-rich protein) genotyping according to the WHO protocols. The genotypic profiles of pre-treatment and day of failure strains were compared to distinguish a recrudescence from a reinfection.[323, 324] For assessment of parasite persistence in follow-up, quantitative polymerase chain reaction (qPCR) targeting PgMET and nested polymerase chain reaction (PCR) targeting *pfmdr1* were also used to screen all samples from day 28 and day 42.[273, 321]

3.15 Outcome measurements

All identified *Plasmodium* species-infecting human from symptomatic and asymptomatic patients were documented. A comparison of microscopy and PCR detection was made. Treatment outcomes from enrolled participants are classified as early treatment failure, late clinical failure, late parasitological failure or adequate clinical and parasitological response (Table 3.2.) according to the latest WHO guidelines.[64]

Table 3.2. Treatment outcomes according to WHO guidelines[64]

Treatment outcomes
Early treatment failure: • Danger signs or severe on day 1, 2 or 3 in the presence of parasitaemia • Parasitaemia on day 2 higher than on day 0, irrespective of axillary temperature • Parasitaemia on day 3 with axillary temperature > 37.5 ^{o}C • Parasitaemia on day 3 > 25% of count on day 0
Late treatment failure: Late clinical failure: • Danger signs or severe malaria in the presence of parasitaemia on any day between day 4 and day 28 (day 42) in patients who did not previously meet any of the criteria of early treatment failure • Presence of parasitaemia on any day between day 4 and day 28 (day 42) with axillary temperature > 37.5 ^{o}C (or history of fever) in patients who did not previously meet any of the criteria of early treatment failure
Late parasitological failure: • Presence of parasitaemia on any day between day 7 and 28 (day 42) and axillary temperature < 37.5 ^{o}C in patients who did not previously meet any of the criteria of early treatment failure or late clinical failure
Adequate clinical and parasitological response • Absence of parasitaemia on day 28 (day 42), irrespective of axillary temperature, in patients who did not previously meet any of the criteria of early treatment failure, late clinical failure or late parasitological failure

Parasite clearance was assessed daily by microscopy at 24-hours intervals following study drug administration until parasites were cleared from blood films. Parasite density was measured at each examination and qPCR was performed as quality control and to determine the proportion of patients remaining parasitaemic at day 1, 2, and 3. Gametocyte prevalence was assessed at enrollment, day 3, day 7, day 14, day 21, day 28, day 35 and day 42. Fever clearance times were determined by measuring axillary temperature on daily visit and as scheduled. Parasite carriage at days 28 and 42 was identified by PCR.

Polymorphisms in *pfcrt* gene, *pfmdr1* gene and *pfk13* propeller on pre-treatment day were evaluated as a predictor tool for therapeutic responses. Molecular data from parasites isolated on follow-up days were also assessed and associated with resistance genotypes.

3.16 Data management

All data collected (clinical notes, laboratory records, supporting data) were recorded in case record forms and could only be accessed by study field manager. Data were subsequently entered into a database with a password protected by a data entry officer and were then reviewed and validated by the investigator. Any missing data were communicated to the site for resolution. The principle investigator was responsible for keeping subject identification code list in a secure location. Access to the database was only available to the study investigator and principle investigator.

3.17 Monitoring and quality assurance

This trial is registered to ClinicalTrials.gov number NCT02325180 and complies with international guidelines for clinical trial reporting. Experienced staff including senior physicians were recruited to perform the study, to monitor participants closely and to ensure that the rights, safety and the wellbeing of the human subjects in the study are in accordance with the study protocol, national protocol, ICH and GCP guidelines. Training session related to study protocol was conducted for all study staff to ensure the expected tasks were performed consistent with the protocols and guidelines.

The study investigator frequently and closely monitored the study site. Trial monitor was appointed to monitor the progress of the study with the objectives of:

- Maintaining the performance of the staff
- Facilitating required work
- Checking the availability of signed informed consents
- Comparing individual records (CRFs) to the source documents
- Ensuring protection of study subjects, compliance with the protocol, and accuracy and completeness of records
- The monitoring schedule were planned prior the start of the study and study investigator and the staff were available at the study site to allow discussion about study progress.

3.18 Reporting and publication of results

At the end of the study, the investigator prepared a final report of the study to be reviewed by advisors and later to be shared with the National Malaria Control Programmes and to the Ministry of Health of Indonesia. Initial results from the study have been presented in several conferences, and was published in a scientific journal. A full report of the clinical study will also be published.

Chapter 4

RESULTS 1

4 RESULTS

Molecular detection of *Plasmodium* spp. in three rural areas of North Sumatera

Some parts of this chapter have been published in a research manuscript (Lubis IND, Wijaya H, Lubis M,Lubis CP, Beshir KB, Sutherland CJ. Contribution of Plasmodium knowlesi to Multispecies Human Malaria Infections in North Sumatera, Indonesia. The Journal of Infectious Diseases. 2017;215(7):1148-1155). Additional material is added here to explain the whole work.

4.1 Introduction

Malaria remains widespread across Southeast Asia. In Indonesia, 2 million cases of malaria are reported each year, with *Plasmodium falciparum* and *P. vivax* the two major cause of illness.[88] Human cases of *P. knowlesi*, a parasite that previously known to infect long-tailed and pig-tailed macaques, have been documented on the islands of Kalimantan and Sumatera[21, 28] but not as a major cause of human malaria as reported in Borneo, Malaysia.[56] The morphological features of this species in the blood stage are similar to *P. falciparum* and *P. malariae* which in routine practice has led to frequent misdiagnosis.[28, 56, 325] Its capability to cause high parasitaemia has also been reported to cause fatal disease.[76] Despite this, a proportion of *P. knowlesi* infections are asymptomatic and submicroscopic across all age groups.[326]

The Ministry of Health of Indonesia has implemented malaria control and elimination efforts since 2009 with 4 distinct stages aiming for the national elimination goal by 2030. Malaria surveillance performed at primary health centres relies on passive case detection by microscopic examination and rapid diagnostic tests (RDTs).[102] These tests are sufficient to detect clinical malaria infection caused by major species.[327] However, identification of less common species is often difficult and low density parasitaemia can lead to underdiagnosis.[328] Modelling of data from low endemicity areas predicts that submicroscopic parasites may contribute 70 to 80% of all malaria infections.[80] Other studies have also shown that these

submicroscopic parasites are infectious to mosquitoes and contribute to ongoing malaria transmission.[196] Hence, the use of routine microscopy and RDT in malaria surveillance fails to detect a substantial proportion of the human reservoir of infection and so may compromise malaria elimination strategies. Deployment of molecular assays, providing excellent sensitivity and specificity, may therefore solve these issues.[51, 329-331]

In this study, we performed intensive malaria screening in 3 regencies of the Province of North Sumatera, western Indonesia. This region is aimed for malaria elimination by 2020. In addition to microscopy, we used established polymerase chain reaction (PCR) assays for detection of *Plasmodium* parasites.[332] However, these tests have limitations for the identification of *P. knowlesi* infection, because the target region of the 18S ribosomal RNA (rRNA) can cross-hybridise with *P. vivax*.[333] Therefore, we developed a novel assay with high sensitivity and highly specific for *P. knowlesi* identification to ensure reliable determination of all *Plasmodium* species, including submicroscopic infection.

4.2 Methods

4.2.1 Study sites and samples collection

A parasitological survey was conducted between January and June 2015 in three selected regencies in North Sumatera province, Indonesia. A total of 47, 10 and 32 temporary clinics were set up in each of Batubara (Figure 4.1.), Langkat (Figure 4.2.) and South Nias (Figure 4.3.) regencies, respectively. Sampling sites were adjusted to the accessibility of community to the local health clinics. There was a good access to healthcare in Batubara, therefore sampling was mainly conducted in the local health clinics with few additional visits in villages and schools (Figure 4.1.). In Langkat and South Nias, geographical locations restraint community access to healthcare, therefore screening of patients was heavily relied on visits to the villages and schools (for South Nias only, Figure 4.2. and 4.3.). Screened individuals were classified into sick individuals visiting the health clinics, household contacts and healthy volunteers, nevertheless this information was not recorded during the survey. Patients with fever or history of fever visiting the outpatients clinics were screened for malaria infection. Healthy individuals visiting the health clinics and in the visited villages who volunteered to be tested and household members of malaria-positive individuals were also included in the malaria testing. During sensitization visits at schools in Batubara and South Nias, parents were asked to fill in questionnaires containing information on history of fever in the past 48 hours of the children. Children with history of fever and healthy children who volunteered were then tested for malaria. Local health clinics were asked to refer patients diagnosed with uncomplicated malaria to our clinics. Finger-prick blood sampling was done to make thick and thin blood films for microscopy examination, RDTs examination (when available), and blood was also spotted on 3MM Whatman filter paper for molecular analysis. Patients confirmed with *P. falciparum* infection by microscopy were subsequently enrolled in a prospective drug efficacy study (Chapter 6) if they fulfilled the inclusion and exclusion criteria (Section 3.10.2.1 and 3.10.2.2). Patients with slide-positive for other *Plasmodium* species were clinically assessed and treated according to national guidelines, but not followed-up. Febrile status in slide-negative individuals were not recorded. Due to the limited numbers of individuals tested with RDTs, only microscopy and PCR results will be presented.

The study was approved by the Ethics committee of the London School of Hygiene and Tropical Medicine (London, United Kingdom; 8504-01), and the Ethics committee of University of Sumatera Utara (Medan, Indonesia; 401/KOMET/FKUSU/2014). Written

informed consent (Appendix 11 to 14) was obtained from adult patients and parents or guardians of enrolled children.

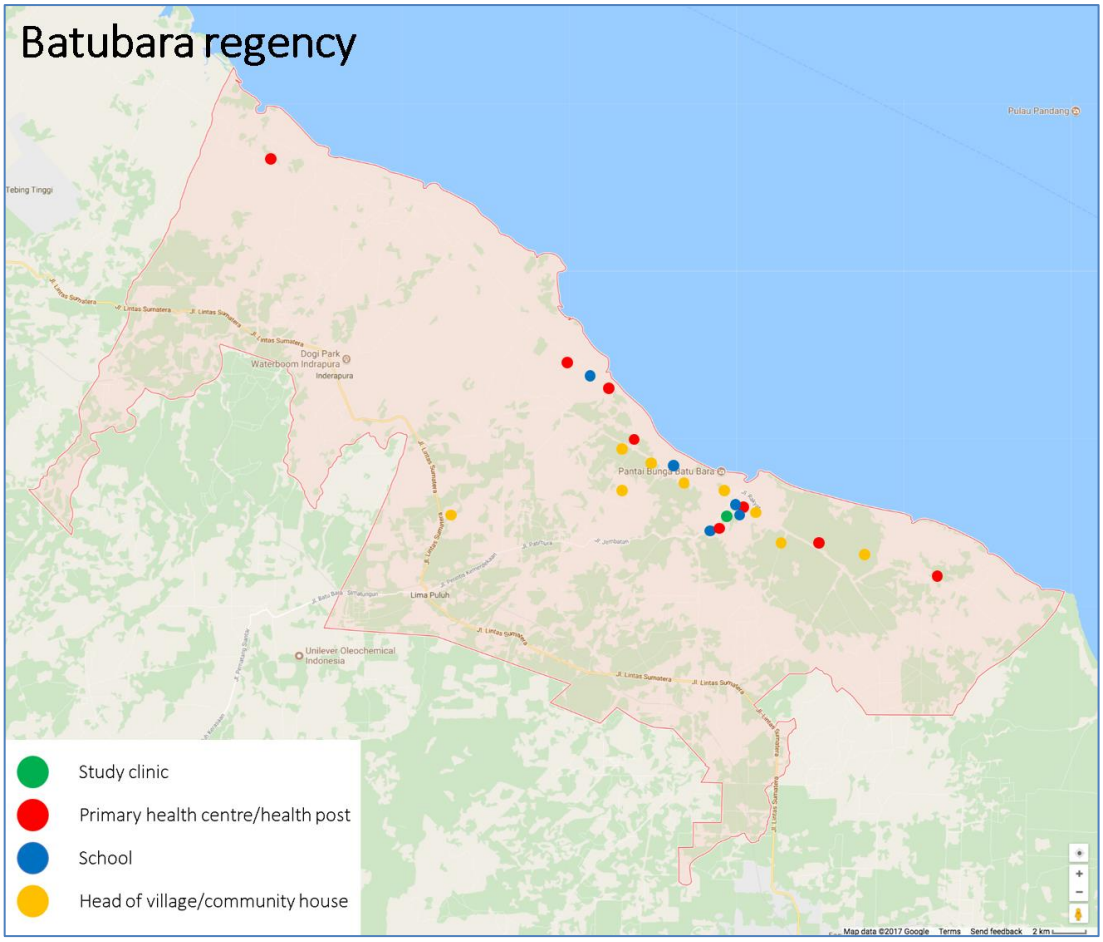


Figure 4.1. Study sites in Batubara regency, North Sumatera province, Indonesia.

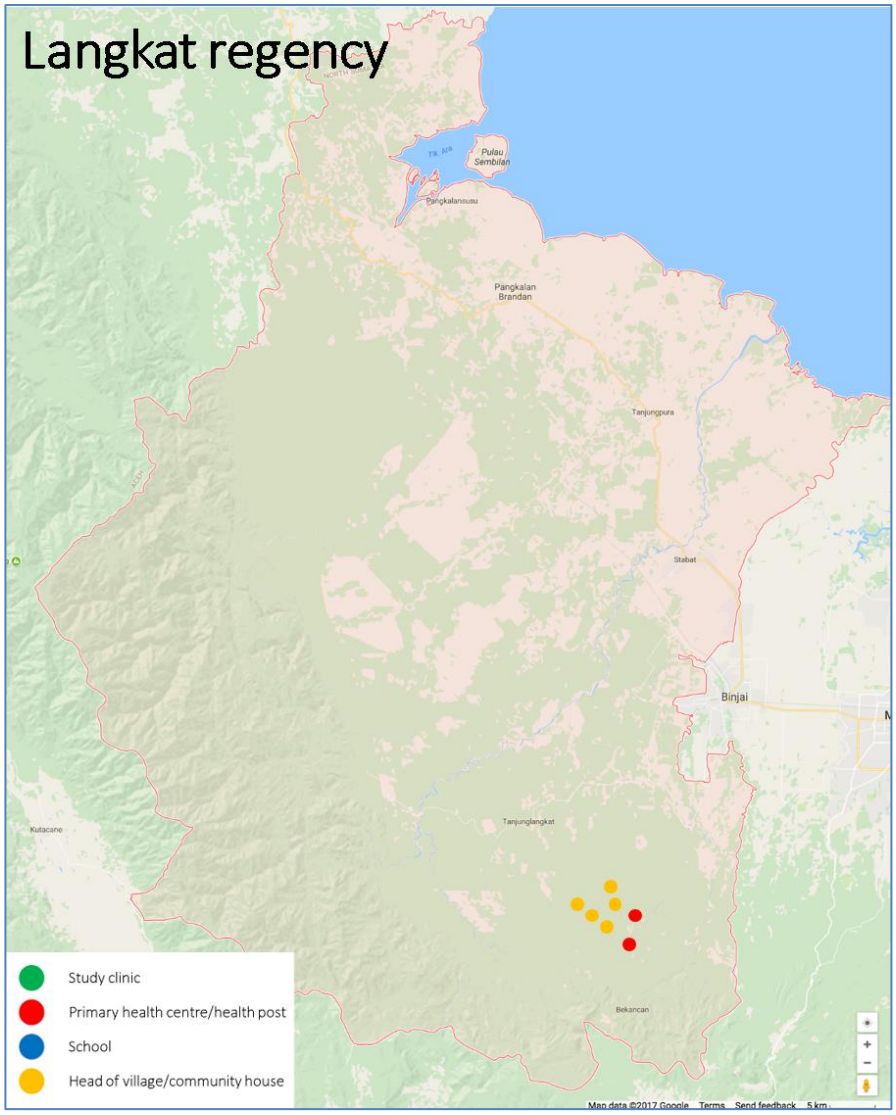


Figure 4.2. Study sites in Langkat regency, North Sumatera province, Indonesia.

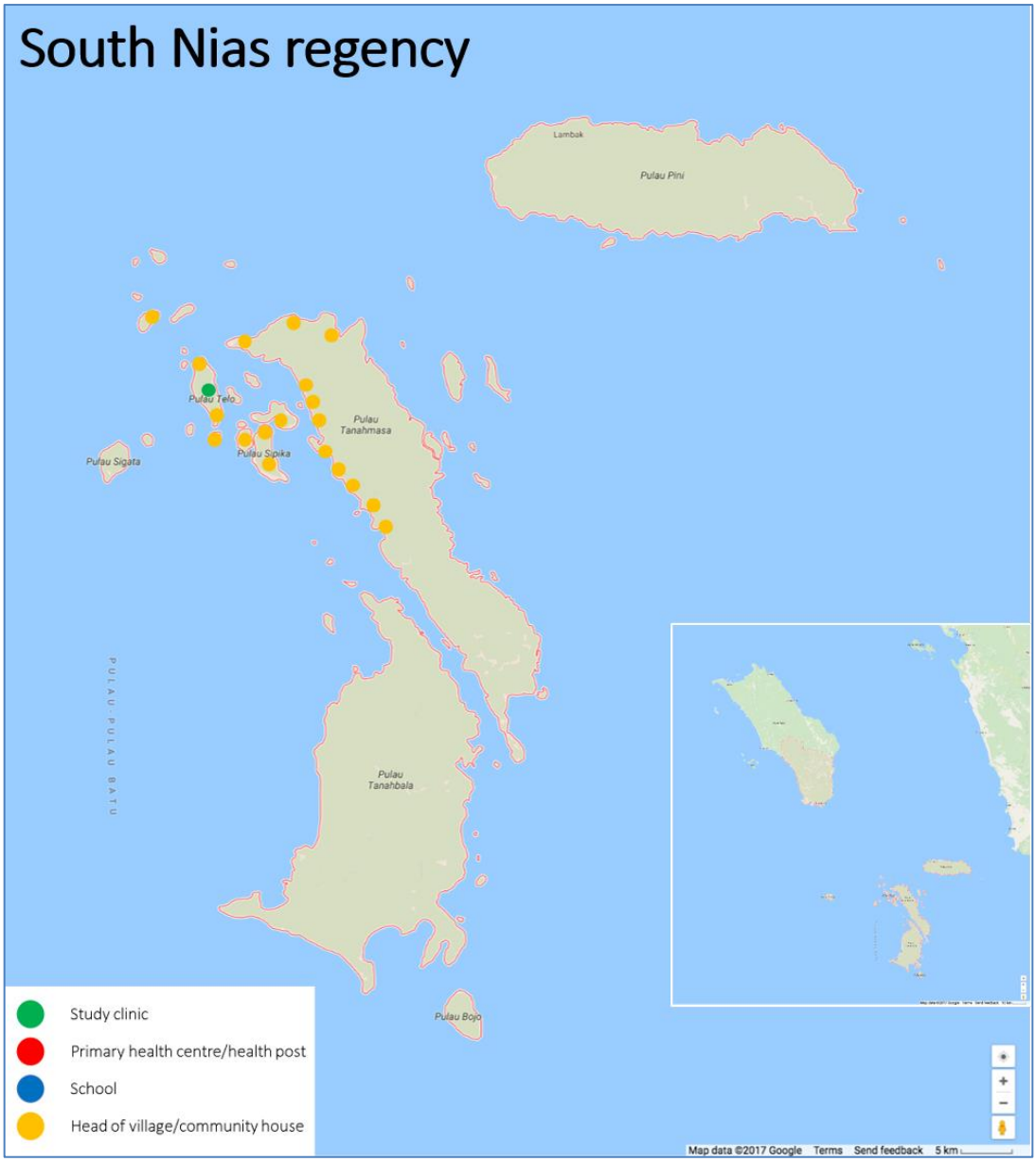


Figure 4.3. Study sites in South Nias regency, North Sumatera province, Indonesia.

4.2.2 DNA extraction

Extraction of parasite DNA from 3MM Whatman filter paper was performed using the Chelex-based method.[320] A dried blood spot of 3 mm in diameter was punched into a well in a 96-deep well plate. The puncher was cleaned with 70% alcohol and flamed before used for the next dried blood spot. One well from each row was left empty as control measure for cross contamination. Dried blood spots were then soaked in 1 ml of 0.5% Saponin overnight. Saponin was dissolved in distilled water and phosphate-buffered saline (PBS) using a magnetic stirrer. After centrifugation, saponin was aspirated from each well and 1 ml of PBS was added to wash the dried blood spots. Samples were incubated for 15 to 30 minutes. During this time, 6% Chelex-100 (BioRad, Richmond, CA) was prepared by adding 6 gr of Chelex-100 to 100 ml of water. Following aspiration of the PBS, 100 µL of 6% Chelex-100 was added into the well and boiled to 100 °C in water block for 10 minutes. After incubation, samples were centrifuged at 10,000 X g for 2 minutes. The supernatant was recovered and placed into a new 96-well plate.

4.2.3 Parasite species identification by rRNA gene amplification

A nested PCR assay targeting the genes encoding *Plasmodium* 18S rRNA was performed on all samples for species determination and detection of additional submicroscopic infection (limit of detection of 1 parasite per µL).[56, 332, 334-336] The primers for nest 1 and nest 2 amplifications are provided in Table 4.1. Reaction mixture for nest 1 included 5 µL of DNA template, NH_4 buffer, 2 mM $MgCl_2$, 200 µM of deoxynucleotides (dNTPs), 250 nM of each primer, and 1 unit of BIOTAQ (Bioline, UK). Cycling conditions for nest 1 amplification were as follows: denaturation at 95 °C for 5 minutes, 25 cycles of 94 °C for 1 minute, 58 °C for 2 minutes and 72 °C for 2 minutes, followed by final extension of 5 minutes at 72 °C. Reaction mixture for nest 2 contained 1 µL of nest 1 product, NH_4 buffer, 2 mM $MgCl_2$, 200 µM of dNTPs, 250 nM of each primer, and 1 unit of BIOTAQ (Bioline, UK). Cycling conditions for nest 2 were as follows: denaturation at 95 °C for 5 minutes, 30 cycles of 94 °C for 1 minute, 58 °C for 2 minutes and 72 °C for 2 minutes, followed by final extension of 5 minutes at 72 °C. Positive controls for *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* spp. and *P. knowlesi*, along with no template control were included in all nested-PCR assays. 8 µL of PCR products from nest 2 amplifications were stained with ethidium bromide and analysed by gel electrophoresis. In the case of no template control yielding positive results, repeat on experiments was performed until negative controls were negative.

Table 4.1. Genus and species-specific primers for nested PCR malaria detection assay[56, 334-336]

PCR	Species		Primer	Sequence	Size (bp)
1st	*Plasmodium* genus	Forward	*rPLU6*	5'- CYTGTTGTTGCCTTAAACTTC -3'	
		Reverse	*rPLU5new*	5'- TTAAAATTGTTGCAGTTAAAACG -3'	
2nd	*P. falciparum*	Forward	rFAL1	5'- TTAAACTGGTTTGGGAAAACCAAATATATT -3'	205
		Reverse	rFAL2	5'- ACACAATGAACTCAATCATGACTACCCGTC -3'	
	P. vivax	Forward	*rVIV1*	5'-CGCTTCTAGCTTAATCCACATAACTGATAC-3'	120
		Reverse	*rVIV2*	5'-ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA-3'	
	P. malariae	Forward	*rMAL1*	5'- ATAACATAGTTGTACGTTAAGAATAACCGC-3'	144
		Reverse	*rMAL2*	5'- AAAATTCCCATGCATAAAAAATTATACAAA-3'	
	P. ovale (classic)	Forward	*rOVA1*	5'- ATCTCTTTTGCTATTTTTTAGTATTGGAGA-3'	800
		Reverse	*rOVA2*	5'- GGAAAAGGACACATTAATTGTATCCTAGTG-3'	
	P. ovale (classic & variant)	Forward	*PadPo*	5'- CTGTTCTTTGCATTCCTTATGC-3'	800
		Reverse	*rOVA2_2v*	5'- GGAAAAGGACACTATAATGTATCCTAATA-3'	
	P. knowlesi	Forward	*Pmk8*	5'- GTTAGCGAGAGCCACAAAAAAGCGAAT-3'	153
		Reverse	*Pmkr9*	5'- ACTCAAAGTAACAAAATCTTCCGTA-3'	

4.2.4 Development of malaria detection PCR assay

To overcome the cross-reactivity between *P. vivax* and *P. knowlesi* commonly reported in PCR assay targeting the 18S rRNA, we designed new molecular assay primarily for detection of all human malaria species targeting a region in mitochondrial *cytochrome b* (*cytb*) gene. The aim of this development is to confirm novel findings of other species than *P. falciparum* and *P. vivax* in this study. *Cytb* has good conservation intraspecies with sequence variation between species.[30] Primers are provided in Table 4.2. PCR mixture for nest 1 contained 5 µl of template DNA, NH_4 buffer, 2 mM $MgCl_2$, 250 µM of dNTPs, 200 nM of each primer, and 1 unit of BIOTAQ (Bioline, UK). For the nested PCR, reaction mixture contained 1 µl of nest 1 product, NH_4 buffer, 2 mM $MgCl_2$, 250 µM of dNTPs, 200 nM of each primer, and 1 unit of BIOTAQ (Bioline, UK). Nest 1 and nest 2 amplifications were run under following conditions: denaturation at 94 °C for 3 minutes, 30 cycles of 94 °C for 30 seconds, 48 °C for 30 seconds and 72 °C for 1 minutes, followed by final extension of 5 minutes at 72 °C. DNA from human *Plasmodium* species including *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale curtisi*, *P. ovale wallikeri*, *P. knowlesi* and simian *Plasmodium* species including *P. cynomolgi*, *P. coatneyi*, *P. inui* and *P. fieldi* were tested using this assay. Standard 2% agarose gel electrophoresis stained with ethidium bromide was used to detect PCR products. Direct sequencing was then performed on PCR products to allow species identification. Geneious (version 8.0.5) and BLAST software were used to identify sequences. A subset of samples were tested using this assay as comparison for the findings by standard PCR assay targeting 18S rRNA.

Table 4.2. Nest 1 and nest 2 primers for *cytochrome b* PCR amplification[30]

PCR		Primer	Sequence	Size (bp)
1st	Forward	PgCytbF1	5'- GAATTATG GAGTGGATGGTG-3	
	Reverse	PgCytbR1	5'- CATCCAATCCATAATAAAGC-3'	
2nd	Forward	PgCytbF1	5'- GAATTATG GAGTGGATGGTG-3	618
	Reverse	PgCytbR2	5'-TTTTAACATTGCATAAAATGG-3'	

4.2.5 Development and validation of a novel highly specific *P. knowlesi* PCR assay

To further validate our findings on *P. knowlesi* isolates, we further designed a new set of primers for a hemi-nested PCR assay based on a conserved region in the gene-family encoding the *P. knowlesi*-specific schizont-infected cell agglutination variant antigens (*sicavar*). The primers used for nest 1 and nest 2 are described in Table 4.3. These newly developed primers generated final amplicons of 228-249 base pairs, encoding 76 – 83 amino acids. Reaction mixture for nest 1 amplification contained 5 µL of DNA template, NH_4 buffer, 2 mM $MgCl_2$, 200 µM of deoxynucleotides (dNTPs), 200 nM of each primer, and 1 unit of BIOTAQ (Bioline, UK). Amplifications for nest 1 was performed under the following conditions: denaturation at 95 °C for 3 minutes, 30 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds and 65 °C for 1 minutes, followed by final extension of 5 minutes at 65 °C. 0.2 µL of nest 1 product was used as a template for the 25 µL reaction mixture of the nest 2 PCR containing NH_4 buffer, 2 mM $MgCl_2$, 200 µM of deoxynucleotides (dNTPs), 200 nM of each primer, and 1 unit of BIOTAQ (Bioline, UK). Cycling conditions for nest 2 amplifications were similar to nest 1 amplifications.

All *Plasmodium* species used to validate the *sicavar* assay was tested using *cytb* PCR assay to confirm the presence of DNA. The *sicavar* assay was then tested against all human malaria parasites (*P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale curtisi* and *P. ovale wallikeri* obtained from Malaria Reference Lab and *P. falciparum* culture parasites at LSHTM), simian malaria parasites (*P. knowlesi*, *P. inui*, *P. cynomolgi*, *P. coatneyi* and *P. fieldi*), clinical *P. knowlesi* isolates obtained from Kapit of Malaysian Borneo and human DNA to assess its specificity. We also determined the limit of detection by using *P. knowlesi* culture of known parasitaemia which was serially diluted in whole human blood and spotted on filter paper to mimic a variety of parasitaemia obtained from field isolates. Initially, 2% parasitaemia was prepared in 50% haematocrit and then diluted accordingly. A total of 40 µL of blood from the series of dilution

was then spotted on filter papers. DNA from these blood spots were extracted with a QIAsymphony automated DNA extraction system resulted in a final volume of 100 µL of DNA, and a total of 5 µL of DNA was used in the experiments. All field isolates were tested for *P. knowlesi* infection using this novel PCR assay. DNA from a subset of positive samples by *sicavar* were validated by direct sequencing using BigDye Terminator v3.1 cycle sequencing kits and analysis on an ABI 3730 Sequencer (Applied Biosystems). Results were aligned and compared to *P. knowlesi* H strain reference using Geneious (version 8.0.5) and BLAST (Basic Local Alignment Search Tool) software.

Table 4.3. Nest 1 and nest 2 primers for *sicavar* PCR amplification

PCR		Primer	Sequence	Size (bp)
1st	Forward	SICAf1	5'-GGTCCTCTTGGTAAAGGAGG-3	
	Reverse	SICAr1	5'- CCCTTTTTGACATTCGTCC-3'	
2nd	Forward	SICAf2	5'-CTTGGTAAAGGAGGACCACG-3'	228-249
	Reverse	SICAr1	5'- CCCTTTTTGACATTCGTCC-3'	

4.3 Results

4.3.1 Validation of *cytb* assay for malaria detection

The *cytb* assay amplified DNA from all human and simian malaria parasites. The products of *cytb* test were sequenced to identify and differentiate *Plasmodium* species. BLAST software was used for species confirmation. Sequences of each species are shown in Figure 4.4. After confirmation of malaria detection using this assay, we tested 11 of our field isolates for comparison with the standard PCR assay for malaria diagnosis (Table 4.4.). *Cytb* gave positive amplifications in all 18S rRNA-positive isolates, but it was only able to identify one of the species present in multiple infections, giving unconvincing diagnosis of mixed *P. vivax* and *P. knowlesi* cases. Interestingly, this test identified 2 additional positive cases missed by the standard PCR test. However, the limited performance of *cytb* to identify multiple species in a single sample led to the development of novel specific assay for detection of *P. knowlesi*. We designed new primers with a target gene of *sicavar*, specific only for *P. knowlesi*. We then used *cytb* assay to confirm the presence of *Plasmodium* species for the validation of *sicavar* assay.

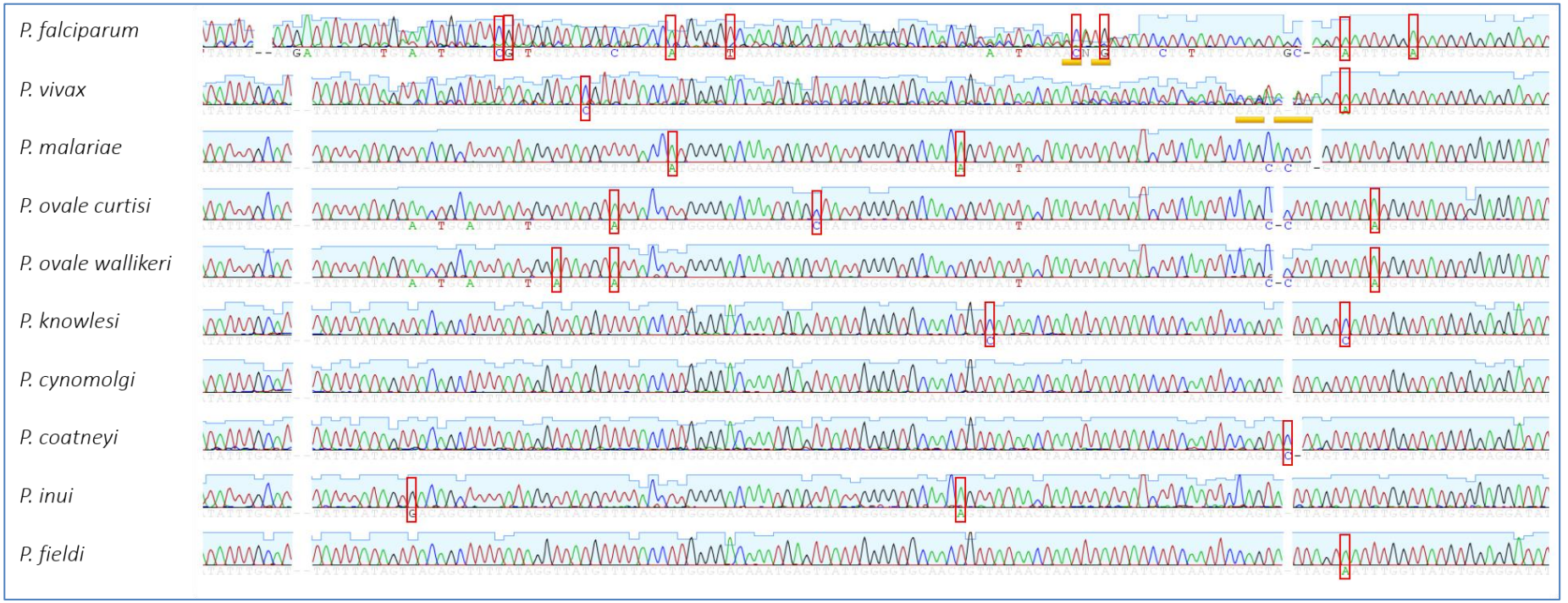


Figure 4.4. Sequences of a portion of *cytochrome b* gene in human and simian *Plasmodium* species.

Table 4.4. Comparison of microscopy and various PCR assays for malaria diagnosis in 11 *Plasmodium* infected individuals from Batubara

ID	Microscopy	PCR assays*		
		18S rRNA	Cytb	SICAvar
BB20001	*P. falciparum*	*P. falciparum*	*P. falciparum*	*P. knowlesi*
BB20002	*P. vivax*	*P. falciparum, P. vivax, P. knowlesi*	*P. vivax*	Negative
BB20003	Negative	*P. vivax*	*P. vivax*	Negative
BB20004	Negative	Negative	*P. vivax*	Negative
BB20005	*P. vivax*	*P. vivax*, *P. knowlesi*	*P. vivax*	Negative
BB20008	Negative	Negative	*P. vivax*	Negative
BB20010	*P. vivax*	*P. vivax*	*P. vivax*	Negative
BB20011	*P. falciparum*, *P. vivax*	*P. vivax*, *P. knowlesi*	*P. vivax*	Negative
BB20012	*P. vivax*	*P. vivax*	*P. vivax*	Negative
BB20013	*P. falciparum*, *P. vivax*	*P. vivax*, *P. malariae*, *P. knowlesi*	*P. vivax*	Negative
BB20019	Negative	*P. vivax*	*P. vivax*	Negative

*For details of PCR assays see Section 4.2.

4.3.2 *Sicavar* assay for *P. knowlesi*-specific identification

The novel *sicavar* assay was found to be specific for *P. knowlesi* and did not amplify other *Plasmodium* species other than *P. knowlesi* (Figure 4.5.). After confirmation of the *sicavar* assay, we determined the limit of detection using a series of dilution of *P. knowlesi* DNA extracted from dried blood spots to mimic samples collected in the field. The limit of detection was estimated as 0.1 parasite per µL of whole blood (Figure 4.6.).

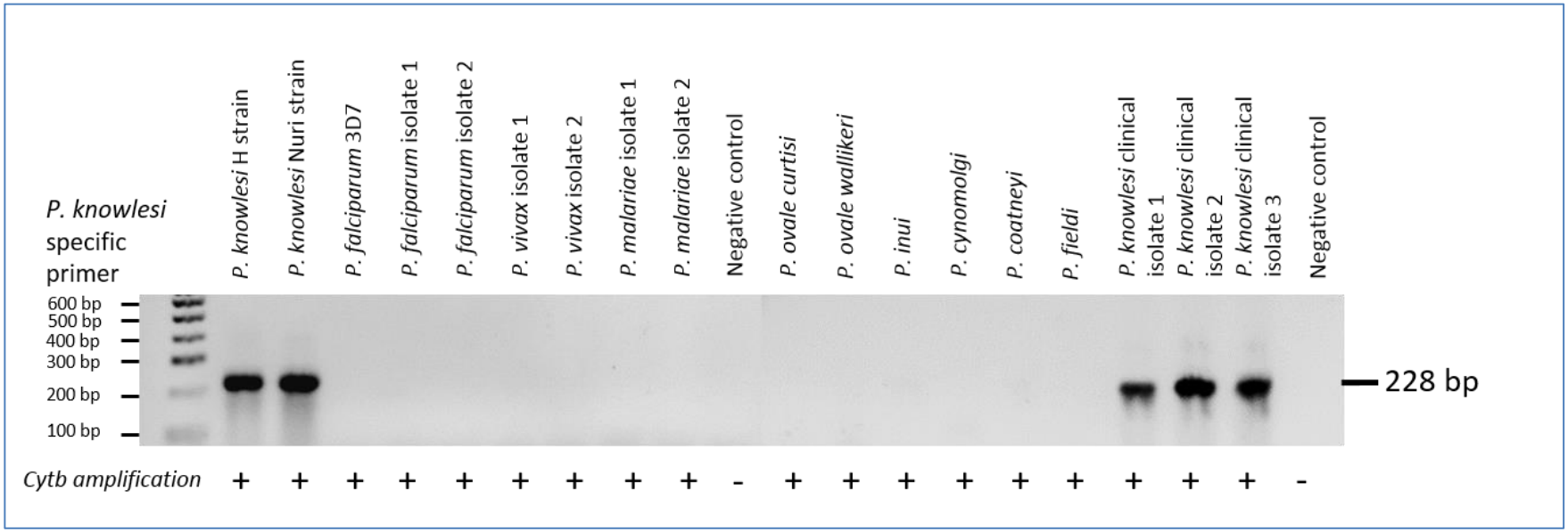


Figure 4.5. Validation of *P. knowlesi* primers targeting *sicavar* against human and simian malaria parasites reported from Southeast Asia. Control DNA of human malaria *Plasmodium* species were from imported cases in the United Kingdom (courtesy of the Public Health England Malaria Reference laboratory); 2 isolates each are shown for *P. falciparum*, *P. vivax* and *P. malariae*. *P. knowlesi* DNA were obtained from clinical *P. knowlesi* patients from Kapit of Malaysian Borneo.

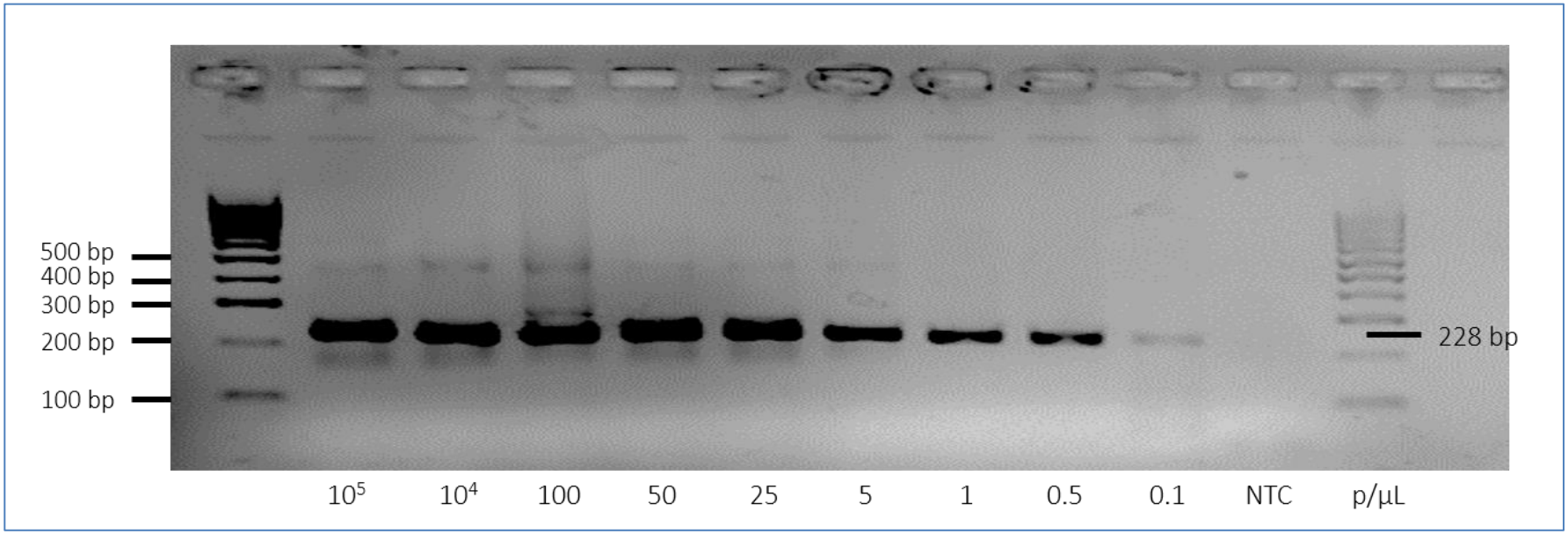


Figure 4.6. Limit of detection of *sicavar* test against series of dilution of *P. knowlesi* DNA. Each DNA sample was tested in triplicates. DNA was obtained from *P. knowlesi* H strain adapted in human blood, DNA was serially diluted in whole human blood and spotted on filter paper. QIAsymphony automated extraction system was used to extract DNA from filter papers.

The *sicavar* targeted primers detected 377 *P. knowlesi* cases, whereas the standard assay targeting 18S rRNA identified only 76 *P. knowlesi* cases, revealing a significantly higher sensitivity compared to the standard assay. Using the two tests, a total of 443 cases were detected but only 10 cases (2.3%) had a positive result in both assays (Table 4.5.). With the *sicavar* results as the definitive diagnosis for *P. knowlesi*, our findings suggested that 66 individuals positive by rRNA nested PCR alone had cross-reactivity with *P. vivax* DNA. Nearly half of all *sicavar* -positive *P. knowlesi* infections were also rRNA amplicon positive for $\geq$ 1 other species, and *P. vivax* being the most common co-infection.

To further confirm our results, we directly sequenced products from 7 *P. knowlesi* isolates detected by SICAvar. The sequences showed high variability (Figure 4.7.), and 2 of the 7 sequences harbored an insert encoding an additional 7 amino acids which was used to probe the current *P. knowlesi* reference genome (http://www.sanger.ac.uk/resources/downloads/protozoa/plasmodium-knowlesi.html).

Table 4.5. Comparison of *sicavar* assay and standard rRNA assay for *P. knowlesi* case detection

Infection detected	Cases, No. (%)			
	18S rRNA assay	SICAvar assay	Any assay	Both assays
Total *P. knowlesi* cases	76 (100)	377 (100)	443 (100)	10 (100)
P. knowlesi monoinfection	42 (55.3)	215 (57.0)	254 (57.3)	3 (30)
P. knowlesi plus *P. vivax*	16 (21.1)	65 (17.2)	77 (17.4)	4 (40)
P. knowlesi plus other *Plasmodium* spp. infections	18 (23.7)	97 (25.7)	112 (25.3)	3 (30)

Figure 4.7. Sequence alignment of representative 120 and 141 nucleotide sequences of *sicavar* amplicons from the peripheral blood DNA of four participants from Batubara (BB), and two each from Langkat (LK) and South Nias (NS). Amplicons were produced by hemi-nested PCR as described in Materials and Methods. Representative amplification products were chosen for this sample, and sequenced directly using amplification primers to prime forward and reverse sequencing reactions. Sequences shown were confirmed in both directions. Two loci from the *P. knowlesi* H strain reference genome (0932000, 0118500) are shown for comparison. Double-peaking was seen in most samples – only the peak with highest amplitude was read for this analysis.

Following this validation, we estimated the contribution of each species to malaria infection in each study sites. To our surprise, we found *P. falciparum*, *P. vivax*, *P. malariae* and *P. knowlesi* to be co-endemic in all the three study sites. *P. falciparum* and/or *P. vivax* are the most abundant species in Langkat and South Nias, as expected. While in Batubara, *P. knowlesi* was the most prevalent (Figure 4.8.).

4.3.3 Parasite carriage

A total of 3731 individuals from Batubara (n=1270), Langkat (n=544), and South Nias (n=1917) were included in the malaria screening. At each regency, 117 (9.2%), 98 (18.0%) and 397 (21.3%) were positive for *Plasmodium* infections by microscopy examination. Three species (*P. falciparum*, *P. vivax* and *P. malariae*) were identified. However, the numbers of confirmed malaria infections decreased with the failure by PCR tests to amplify DNA from stored blood spots from individuals deemed positive by microscopy in the field. Thus, the total numbers of malaria positive cases became 93 (8.1%), 74 (13.6%) and 169 (9.1%). Poor specificity of microscopy was particularly observed in South Nias (Table 4.6.) Nevertheless, the successful detection of submicroscopic infection meant PCR-confirmed parasite carriage was estimated as 25.2%, 33.5% and 35.3% of all tested individuals (Table 4.7.). All *Plasmodium* species with the exception of *P. ovale* spp. were detected by a combination of the rRNA gene and *sicavar* PCR assays.

Table 4.6. Odds ratio for PCR negative microscopy positive results according to study sites and parasite density

	Odds Ratio	95% Confidence Interval	*P* Value
Study Site			
Batubara	Reference		
Langkat	1.16	0.90 – 1.50	0.24
South Nias	1.62	1.36 – 1.93	**0.001**
Parasite density (p/µL)			
< 100	Reference		
101 – 250	1		
251 – 500	0.95	0.13 – 6.83	0.96
501 – 1000	1.29	0.12 – 14.53	0.84
> 1000	1		

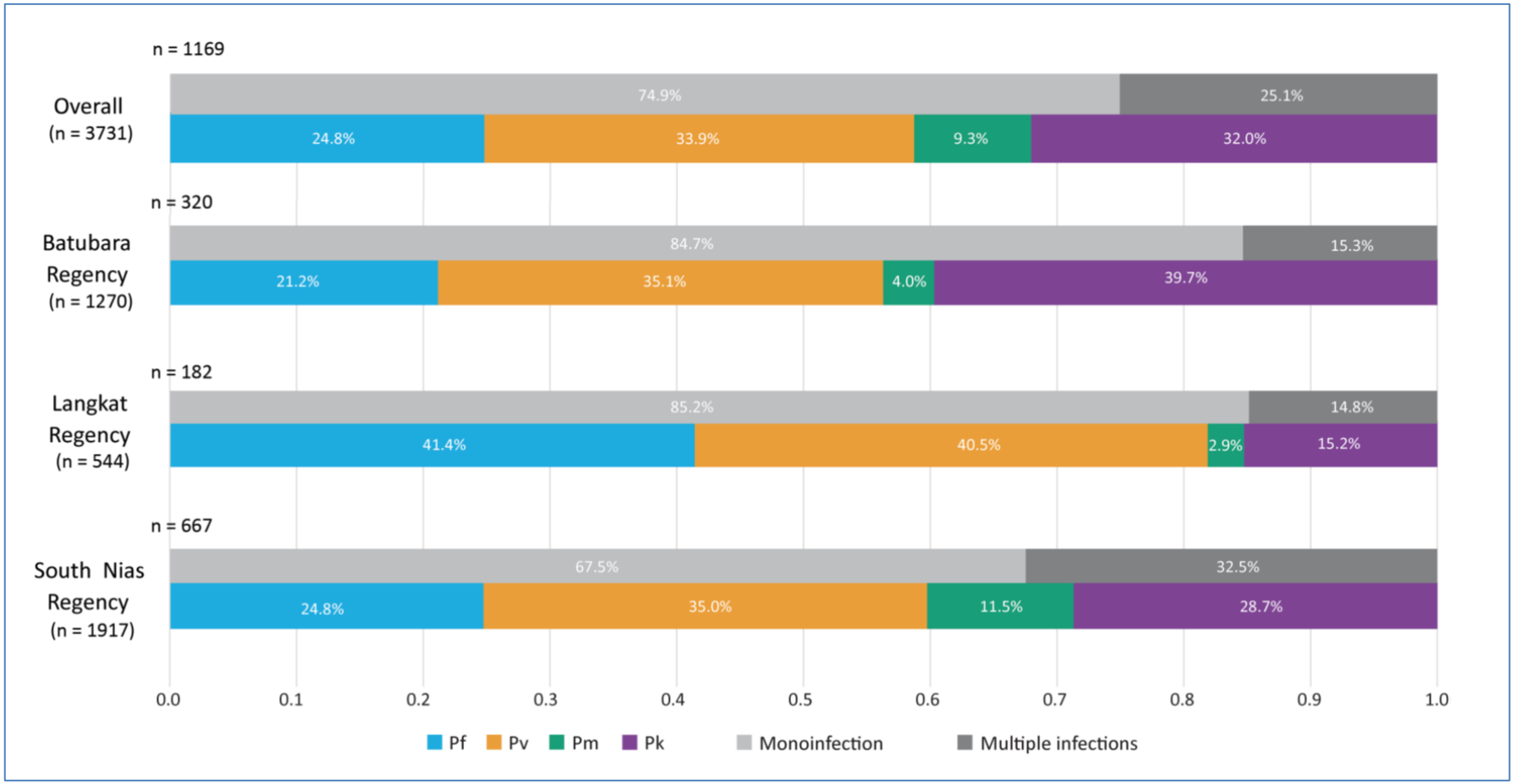


Figure 4.8. Proportion of *Plasmodium* species and multiplicity of infections by regency. Numbers on the left of the bar represent the numbers of individual tested, and numbers above the bar represent the total numbers of positive cases. Abbreviation: Pf=*P. falciparum*, Pv=*P. vivax*, Pm=*P. malariae*, Pk=*P. knowlesi.*

Table 4.7. Submicroscopic infections in 1169 participants with positive PCR results for *Plasmodium* spp.

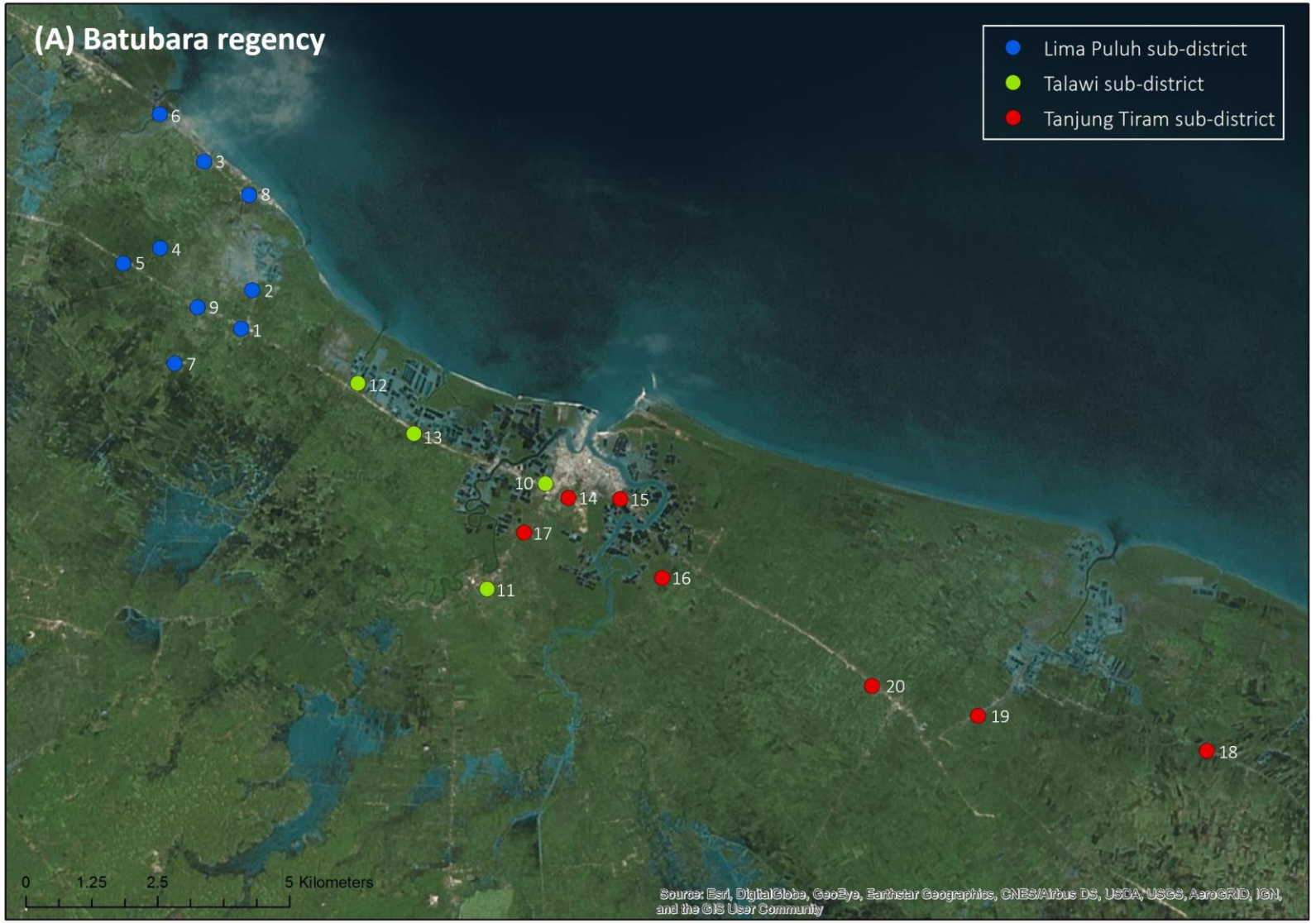
Infection type	Participants, No. (%)	
	All PCR* positive	PCR* positive and microscopy negative
All *Plasmodium* infections	1169 (31.3)	833 (71.3)
Plasmodium falciparum	247 (6.6)	165 (14.1)
Plasmodium vivax	335 (8.9)	227 (19.4)
Plasmodium malariae	40 (1.1)	35 (2.9)
Plasmodium knowlesi	254 (6.8)	220 (18.8)
Mixed infections	293 (7.9)	186 (15.9)
No. of species by PCR		
1	876 (74.9)	647 (77.7)
2	256 (21.9)	163 (19.6)
3	35 (2.9)	21 (2.5)
4	2 (0.2)	2 (0.2)
PCR positive by regency		
Batubara	320/1270 (25.2)	227/320 (70.9)
Langkat	182/544 (33.5)	108/182 (59.3)
South Nias	667/1917 (34.8)	498/667 (74.7)

*For details of PCR assays see Section 4.2.

With the increased numbers of infection detected by molecular tests, we found that living in South Nias increased the risk of infection to *P. vivax* (OR 1.49, 95% CI 1.23-.179, *P*=0.001), *P. malariae* (OR 5.05, 95% CI 3.12-8.52, *P*=0.001), and *P. knowlesi* (OR 1.43, 95% CI 1.17-1.76, *P*=0.001). While risk of acquiring *P. falciparum* infection only increased in residents of Langkat (OR 1.81, 95% CI 1.39-2.35, *P*=0.001). We found there was no increased risk of infection with age. Our particular interest was the increased risk of *P. knowlesi*, as to our knowledge this is the first study outside Malaysian Borneo to report a high burden of *P. knowlesi* infection in humans. Thus, we tried to map geographically the contribution of this species at the village-level (Table 4.8). In this effort, we decided to focus on Batubara regency due to less population movement in this area (Figure 4.9.A.). We found that incidence of *P. knowlesi* infections among individuals residing in urban areas (Figure 4.9.C) was similar to those living in the rural settlements (Figure 4.9.B)

Table 4.8. Proportion of *P. knowlesi* infection among all tested individuals according to sampling sites in Batubara regency

Site ID	District	Site	Proportion of *P.* knowlesi, n (%)
1	Lima Puluh	Puskesmas Kedai Sianam	5/19 (26.3)
2		Guntung	12/159 (7.5)
3		Dusun 1 Perupuk	8/80 (9.1)
4		Balai Desa Titi Putih	5/39 (11.4)
5		Titi Putih	2/77 (2.5)
6		Perupuk	18/153 (10.5)
7		Pasir Permit	0/8 (0)
8		SMPN 4 Lima Puluh	2/51 (3.8)
9		SDN 016517 Guntung	6/38 (13.6)
10	Talawi	Indrayaman	6/29 (20.7)
11		Puskesmas Labuhan Ruku	2/21 (9.5)
12		Dahari Selebar	13/175 (7.4)
13		Mesjid Lama	0/11 (0)
14	Tanjung Tiram	Puskesmas Tanjung Tiram	47/192 (24.5)
15		Bagan Dalam	4/31 (12.9)
16		Sentang	3/24 (12.5)
17		Posko Tanjung Tiram	10/47 (21.3)
18		Pustu Bagan Baru	0/10 (0)
19		Tali Air	1/14 (7.1)
20		Puskesmas Ujung Kubu	3/27 (11.1)
21	Medang Deras	Pagurawan	0/1 (0)


(A) Batubara regency
Lima Puluh sub-district
Talawi sub-district
Tanjung Tiram sub-district
6
3
8
4
5
2
9
1
7
12
13
10
14
15
17
16
11
20
19
18
0
1.25
2.5
5 Kilometers
Source: Esri, DigitalGlobe, GeoEye, Earthstar Geographics, CNES/Airbus DS, USDA, USGS, AeroGRID, IGN, and the GIS User Community


(B)
Proportion of P. knowlesi infections
P knowlesi negative
P knowlesi positive
0
0.5
1
2 Kilometers
Source: Esri, DigitalGlobe, GeoEye, Earthstar Geographics, CNES/Airbus DS, USDA, USGS, AeroGRID, IGN, and the GIS User Community


(C)
Proportion of P. knowlesi infections
P knowlesi negative
P knowlesi positive
0 0.5 1 2 Kilometers
Source: Esri, DigitalGlobe, GeoEye, Earthstar Geographics, CNES/Airbus DS, USDA, USGS, AeroGRID, IGN, and the GIS User Community

Figure 4.9. (A) Sampling sites in Batubara regency; (B) Proportion of *P. knowlesi* infection among tested individuals according to sampling sites in Lima Puluh and Talawi sub-districts; (C) Proportion of *P. knowlesi* infection among tested individuals according to sampling sites in Tanjung Tiram sub-district.

4.3.4 Carriage of submicroscopic infection

PCR analysis of 1169 infected individuals revealed the vast majority of the infections (71.3%) were submicroscopic (Table 4.7.). Among these submicroscopic infections, 77.7% (647 of 833) were single infection predominated by *P. vivax* (27.3%, *n*=227) and *P. knowlesi* (26.4%, *n*=220) (Table 4.7.). We found adults to be more likely to carry submicroscopic infection compared to older children (OR 1.81, 95% CI: 1.38-2.38) and younger children (OR 1.75, 95% CI: 1.15-2.64). While mean age of individuals with patent infection was 18.0 years (95% CI, 16.2 – 19.7 years; P<0.001, 2-sided *t*-test). Submicroscopic cases were also more likely to occur in Batubara (OR 1.67, 95% CI: 1.14 – 2.45) and South Nias (OR 2.02, 95% CI: 1.43-2.85) than in Langkat. However, infection with multiple species was not significantly associated with increased age among malaria positive individuals (*P*=0.66, 2-sided *t* test).

Table 4.9. Detection of *Plasmodium* species by microscopy and nested PCR*

	Microscopy	Detection by Nested PCR																
		Pf	Pv	Pm	Pk	Pf Pv	Pf Pm	Pf Pk	Pf Pv Pm	Pf Pv Pk	Pf Pm Pk	Pf Pv Pm Pk	Pv Pm	Pv Pk	Pv Pm Pk	Pm Pk	Neg	*Total*
Batubara Regency	Pf	18	-	-	-	3	-	5	-	-	-	-	-	-	-	-	8	*34*
	Pv	1	37	-	4	3	-	-	-	3	-	-	-	11	-	-	15	*74*
	Pf Pv	1	3	-	-	2	-	-	-	-	-	-	-	1	1	-	1	*9*
	Pm	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Neg	34	53	10	110	4	-	5	-	-	-	-	3	7	-	1	926	*1153*
	Total	*54*	*93*	*10*	*114*	*12*	*-*	*10*	*-*	*3*	*-*	*-*	*3*	*19*	*1*	*1*	*950*	***1270***
Langkat Regency	Pf	18	3	-	3	2	-	-	-	-	-	-	-	-	-	-	15	*41*
	Pv	3	24	-	1	5	-	-	-	-	-	-	-	5	-	-	7	*45*
	Pf Pv	4	1	-	-	1	-	-	-	-	-	-	-	3	-	-	2	*11*
	Pm	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	*1*
	Neg	45	34	2	16	5	2	1	-	1	-	-	-	1	-	1	338	*446*
	Total	*70*	*62*	*3*	*20*	*13*	*2*	*1*	*-*	*1*	*-*	*-*	*-*	*9*	*-*	*1*	*362*	***544***
South Nias Regency	Pf	26	22	3	18	7	2	14	1	1	2	-	2	12	2	2	154	*268*
	Pv	8	16	-	7	2	1	1	-	2	-	-	1	1	1	1	64	*105*
	Pf Pv	3	2	1	1	2	-	-	-	-	1	-	2	2	-	-	12	*26*
	Pm	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Neg	86	140	23	94	25	4	26	-	8	3	2	25	34	9	19	1020	*1518*
	Total	*123*	*180*	*27*	*120*	*36*	*7*	*41*	*1*	*11*	*6*	*2*	*30*	*49*	*12*	*22*	*1250*	***1917***

Pf=*P. falciparum*, Pv=*P.vivax*, Pm=*P.malariae*, Pk=*P.knowlesi*

*Nested PCR targeting the 18 ssu rRNA gene (plus *sicavar* gene for *P.knowlesi* infection)

Table 4.10. Age and sex of participants with positive PCR results for *Plasmodium* spp.

Age and sex by *Plasmodium* species	PCR positive, No. (%)
Parasite carriage by age group, y	
P. falciparum	
<5	24 (9.7)
5-14	91 (36.8)
>15	132 (53.4)
P. vivax	
<5	41 (12.2)
5-14	143 (42.7)
>15	151 (45.1)
P. malariae	
<5	4 (10.0)
5-14	18 (45.0)
>15	18 (45.0)
P. knowlesi	
<5	28 (11.0)
5-14	96 (37.8)
>15	130 (51.2)
Parasite carriage among female participants	
P. falciparum	137 (55.5)
P. vivax	171 (51.0)
P. malariae	15 (37.5)
P. knowlesi	129 (50.8)

4.4 Discussion

In this study, we reported contribution of malaria infections in 3 regencies in North Sumatera province. This study combined microscopy and PCR for detection of *Plasmodium* parasites among febrile and nonfebrile residents. PCR identified 1169 individuals harboring *Plasmodium* species, much higher compared to 612 cases identified by microscopy. *P. falciparum* and *P. vivax* are the two major contributors for malaria infection as reported in regional malaria reports.[312] However, using molecular tests we detected 4 species to be circulating: *P. falciparum*, *P. vivax*, *P. malariae* and *P. knowlesi*.

In Indonesia, the national malaria control programmes focus on case management through passive surveillance at primary health centres, deploying microscopy or RDTs to detect malaria cases to be treated.[102] Microscopy is the gold standard to measure parasite infection and often used as the point-of care diagnostic.[337] However, the quality of examination depends highly on the microscope, staining reagents and the skills of the technician.[338] When performed by an experienced microscopist, the limit of detection is around 50 parasites per microliter and sensitivity and specificity of 95% and 98% can be achieved.[338] In our study, microscopy identified many *Plasmodium* spp. infections but lacked accuracy in distinguishing among the 4 species, as previously reported in Malaysia.[339] Although personnel differed between sites, identification of non-major species was still problematic in all sites. Microscopy only identified 1 case of *P. malariae*, while *P. knowlesi* was left unrecognized. Poor specificity of microscopy was particularly common in South Nias, leading to a number of false-positives, whereas specificity was better at the other two sites. Samples collection in South Nias involved sea journeys therefore transfer of samples and storage conditions were not optimal and might have resulted in a loss of PCR sensitivity.[340]

Innovation of RDTs has also advanced the methods for malaria diagnosis and provides new techniques for detection of uncomplicated symptomatic malaria and severe malaria.[341] These tests have detection limit ranging from 100 to 200 parasites per microliter[337], and are based on *P. falciparum* histidine-rich protein 2 and/or *Plasmodium* parasite lactate dehydrogenase (pLDH) monoclonal antibodies.[328] Thus, these tests are limited for detection of *P. falciparum* and *P. vivax* only, although high sensitivity of pLDH-based RDTs has been reported in severe cases caused by *P. knowlesi*, these tests have limited capacity for distinguishing the non-falciparum species.[328, 341] In this study, only 28.7% of the infections detected by PCR were also identified by either one or both of the conventional tests,

suggesting the presence of many subpatent and asymptomatic infections. Therefore, both conventional methods were satisfactory for detection of symptomatic cases requiring treatment, but provide limited sensitivity to detect submicroscopic infections. Because these submicroscopic *Plasmodium* carriage is associated with subsequent transmission to mosquitoes, these tests therefore are not adequate tools for malaria elimination and control activities.[196]

Molecular tests are highly sensitive and specific, and provide a greater sensitivity to detect low density infections missed by microscopy or RDT. Molecular techniques for detection of human malaria infections are well established[342], however the current test used to identify *P. knowlesi* infection is not yet optimal due to cross-amplification of *P. vivax* with ribosomal gene PCR.[56, 333] In this study, we initially developed a PCR-sequencing approach to detect human malaria infection aiming for confirmation of *P. knowlesi cytb* DNA. This test provides us with satisfactory sensitivity.[30] But, in Indonesia where mixed infections are frequent, this test is limited with the capacity to only detect one species. In order to meet this challenge we developed a novel assay targeting *sicavar* gene, which gives a greater sensitivity for *P. knowlesi* identification and detected an unexpectedly large number of *P. knowlesi* infection in our study. *Sicavar* genes encode an antigen family unique to *P. knowlesi* with estimated > 100 members, including both multiexon and truncated forms, randomly distributed across all 14 chromosomes.[343] *Sicavar* proteins undergo antigenic variation in the course of single infection[344, 345] and are likely to play a key role in maintaining chronic parasitaemia in semi-immune hosts. Sequencing of a handful of *sicavar* amplicons from our samples confirmed nucleotide diversity in our short target sequence, double peaking indicative of multiple loci being amplified in some cases, and distinguished a variant form with a 7-amino acid insert. Probing the *P. knowlesi* reference genome with these 2 forms generated many hits with both length variants, including both types 1 and 2. Therefore, despite moderate numbers of *P. knowlesi* coinfections with *P. vivax* in the present study, we can confidently report our assay is performing as hoped and is a useful tool for identifying *P. knowlesi* infections among complex mixtures of *Plasmodium* species.

P. knowlesi infection has been widely recorded in Southeast Asian countries, but the largest number of cases were reported from Malaysian Borneo.[325] In Indonesia, it was initially reported in a handful of cases among gold miners in Kalimantan, eastern Borneo.[21, 226] More recently, more cases were identified in Aceh province, on the northwest of Sumatera.[28] This latter study used loop-mediated isothermal amplification (LAMP) in a passive and reactive

case detection to detect malaria cases and identified a total of 20 *P. knowlesi*,, 15 *P. vivax*, and 8 *P. falciparum* among 1532 tested individuals, mostly symptomatic.[28] This contrasts to our findings of higher parasite burden in the population, and of a significant proportion of subpatent and asymptomatic infections, suggesting inter-province differences.

Aceh is a neighboring province of North Sumatera, however has much lower malaria transmission intensity and is aimed for the elimination goal by 2015.[88] This pattern however is similar to those found in Malaysian Borneo where *Plasmodium* species other than *P. knowlesi* have decreased and *P. knowlesi* is now the most common infecting species.[55, 325] In North Sumatera, *P. falciparum* and *P. vivax* are still widely distributed, as shown in our data. Thus, acquired immunity to these human parasites may protect individuals subsequently infected with *P. knowlesi*[346], although asymptomatic infections have also been reported in Sabah Malaysia.[326] North Sumatera's natural forests have been affected by deforestation in the last few years. Residents of rural districts may live in close proximity to semiforested, forested and plantation areas, with a high likelihood of forest exposure, but may not have adequate access to health facilities and antimalarial medication. Our data shows that there is no difference in *P. knowlesi* incidence between sampling sites in Batubara.

Despite the aim to achieve malaria elimination by 2020 in Sumatera, our findings demonstrate that our study sites still pose the risk of malaria infection to humans especially in South Nias. *P. falciparum* still contributes to one-fifth of all malaria cases, while *P. vivax* is slightly more prevalent. Interestingly, our findings also demonstrate a substantial number of *P. knowlesi* infections suggesting that *P. knowlesi* was just as common as the other two main *Plasmodium* species. Macaques were present at all sites, and communities shared established risk factors for malaria transmission. Many *P. knowlesi* infected individuals carried multiple *Plasmodium* infections, in contrast to areas in Malaysian Borneo where *P. falciparum* and *P. vivax* are now very scarce.[325, 326] Nevertheless, sampling was not done appropriately to assess transmission intensity (lack of randomisation strategies and limited records of febrile status in tested individuals), therefore our results do not represent the true prevalence of each malaria species but to show the distribution of four *Plasmodium* species in North Sumatera. Multiple-species infections in our study were equally distributed across all age groups with both female and male subjects exposed to a similar risk of infection, whereas submicroscopic infections were more common in older individuals, suggesting a role for acquired immunity.[346] The observation of asymptomatic *P. knowlesi* infections in our study is consistent with recent findings in Malaysia[326] but does not necessarily support the occurrence of human-mosquito

transmission of *P. knowlesi*. Our data also suggest cross-protection among the 4 human *Plasmodium* species that maintain overall parasite density at low levels.[346]

Our study demonstrated the presence of 4 *Plasmodium* species in North Sumatera including *P. knowlesi*. The latter species is widely circulating in all three study sites, and contributes to this in a high proportion as submicroscopic infections. There is a need to investigate human risk to this infection, and for the national malaria programmes to include in malaria guidelines the recommendation that microscopist are trained to identify *P. knowlesi* infections in Indonesian clinics. Molecular detection of infection is also needed, to strengthen control and elimination programmes by accurately defining the true extent of the malaria reservoir, so as to achieve the current goal of elimination by 2020.[85]

Chapter 5

RESULTS 2

5 RESULTS

Markers of antimalarial drug resistance in *Plasmodium falciparum* parasites in North Sumatera

5.1 Introduction

Successful malaria elimination strategies depend heavily on the effectiveness of the first-line antimalarials drugs. Failures with previously recommended therapies; chloroquine and sulfadoxine-pyrimethamine, had compromised malaria control strategies in many malaria endemic countries and contributed to a significant increase in morbidity and mortality.[160] Today, artemisinin combination therapies (ACTs) are the mainstay of treatment for uncomplicated *Plasmodium falciparum* infection, relying on the combination of fast-acting artemisinin and long protection by its partner drug to delay the development of resistance to this combination.[347] Nevertheless, artemisinin resistance, characterised by slow parasite clearance has been reported in Cambodia, Thailand, Vietnam, Myanmar, Laos and China.[120, 203, 252-259] These artemisinin-resistant parasites have been postulated to facilitate the development of resistance to the partner drugs leading to a substantial decreased of ACT efficacy.[118, 348]

The progression to clinical resistance or the spread of resistance parasites may be prevented by the implementation of regular monitoring on drug resistance markers of *P. falciparum* parasites. Resistance to aminoquinolines is known to be mediated by the putative transporter *pfcrt* [269], with mutation at codon 76T is associated with resistance to chloroquine and amodiaquine.[349] However, the degree of resistance to these drugs is modulated by additional mutations from other genes including the gene encoding P-glycoprotein H1, *pfmdr1*. Mutation of N86Y of *pfmdr1* has been associated with greater resistance to chloroquine and amodiaquine, but in contrast linked to an increased sensitivity to artemisinin,

lumefantrine and mefloquine.[273, 350] Furthermore, some polymorphisms in the *P. falciparum* K13-propeller domain have also been associated with slow-clearing infections and become the marker for artemisinin resistance.[268] Resistance markers for partner drugs of ACT such as piperaquine have also been established, with amplifications in *plasmepsin* gene 2-3 copy numbers and reduced copy numbers in *pfmdr1* were shown to be associated with reduced susceptibility to this drug.[295, 351] Given the availability of these molecular markers today, the use of these tools in epidemiological studies is essential for monitoring antimalarial sensitivity and more importantly allowing real-time measurement of the parasite genetic changes before treatment starts failing.

ACT has been used in Indonesia since 2004 after failures using the previous first-line antimalarial, chloroquine. Artesunate-amodiaquine (ASAQ) was initially deployed, but in 2007 two ACTs were recommended, ASAQ continued to be used for western Indonesia and dihydroartemisinin-piperaquine (DP) was deployed for eastern Indonesia.[86] However, treatment failures with ASAQ were frequently documented which led to another drug policy change in 2012 to the wide deployment of DHP across the country. *In vivo* studies using ASAQ for falciparum malaria demonstrated poor clinical efficacies in Central Java, Papua and Sumatera[171, 176-178], with one study showed PCR-corrected efficacy as low as 80% even prior to the adoption of ASAQ to the national recommendation.[171] The lack of information on parasite polymorphisms in these studies nevertheless hindered the efforts to design the accurate strategy for alternative treatment. Of great concern is that artemisinin-mutant parasites are now spreading across Southeast Asia, and with the history of less responsive to previous ACT treatment in this region, there is an urgent need to further investigate the genetic resistance of *P. falciparum* parasites in western Indonesia.

5.2 Methods

5.2.1 Study sites and samples collection

Samples were from a parasitological survey in Batubara, Langkat and South Nias regency in North Sumatera province, collected between January and June 2015. Description of the study sites and methods for sampling are described in details in Chapter 3 and 4. A total of 3731 participants were screened for *P. falciparum* infection by microscopy and nested polymerase chain reaction (PCR). Individuals with *Plasmodium* parasites identified by microscopy were treated with the standard 3-dose regimen of dihydroartemisinin-piperaquine (DP) or 6-dose regimen of artemether-lumefantrine (AL) plus primaquine, given according to their body weight. The study was approved by the ethics committee of the London School of Hygiene and Tropical Medicine (London, United Kingdom; 8504-01) and the ethics committee of the University of Sumatera Utara (Medan, Indonesia; 401/KOMET/FK USU/2014). Written informed consent (Appendix 11 to 14) was obtained from adult patients and parents or guardians of enrolled children.

5.2.2 Characterisation of parasite polymorphisms

Parasite DNA was isolated from dried blood spots using the Chelex-based method.[320] All samples were screened by nested PCR assays targeting the 18S ribosomal RNA (rRNA) and *pfmdr1* gene.[273, 332] *P. falciparum* isolates detected by polymerase chain reaction (PCR) were subsequently genotyped for all loci of interest in the *pfcrt* gene, *pfmdr1* gene and *pfk13* propeller domain.

Polymorphisms in the *pfcrt* gene, encoding the *P. falciparum* chloroquine resistance transporter, were determined using multiplex qualitative PCR (qPCR) Rotor-gene® Q thermocycler (Corbett Research, Australia) to detect the most common haplotypes at codons 72 to 76, as previously described.[270, 352] Samples were initially run in nest 1 PCR to enable detection of low density parasites. Reaction mixture for nest 1 contained 5 µL of DNA template, NH_4 buffer, 2 mM $MgCl_2$, 200 µM of deoxynucleotides (dNTPs), 400 nM of each forward and reverse primer (Table 5.1.) and 1 unit of BIOTAQ (Bioline UK). PCR was performed with an initial denaturation step at 95 °C for 5 minutes, followed by 35 cycles of 95 °C for 30 seconds, 56 °C for 30 seconds, 62 °C for 1 minute, and final extension of 62 °C for 5 minutes.

Nest 1 product were subsequently used as template for qPCR for *pfcrt* genotype identification. Double-labelled probes designated for haplotypes CVMNK, CVIET and SVMNT with a reported dye at the 5' end and a quencher moiety at the 3' end were used (Table 5.1.). The wild-type *pfcrt* haplotype encoding CVMNK at codons 72-76, and the mutant haplotypes encoding variants of *pfcrt*, CVIET and SVMNT.[270] Primer sets used in this assay are provided in Table 5.1. Reaction mixture for this qPCR assay contained 5 µL of nest 1 product, NH_4 reaction buffer, 5.5 mM $MgCl_2$, 600 µM of dNTPs, 600 nM of each primer, 200 nM of each probe and 1 unit of BIOTAQ (Bioline, UK). Cycling conditions were as follows: denaturation at 95 °C for 6 minutes, 45 cycles of 95 °C for 15 seconds, 55 °C for 1 minute. DNA from 3D7, Dd2 and 7G8 clones were used to represent *pfcrt* haplotypes CVMNK, CVIET and SVMNT, respectively. A single reaction was prepared for each field isolate for qualitative measurement. Thresholds for each probe were set manually using the positive and negative controls. Genotypes of each sample were determined according to which of the reported dye signals accumulated.

Table 5.1. Primers and probes for *pfcrt* genotyping[269, 270]

Assay	Primer	5' Fluorophore	Sequence	3' Quencher
PCR Nest 1	P1		5'-CCG TTA ATA ATA AAT ACA CGC AG-3'	
	P2		5'-CGG ATG TTA CAA AAC TAT AGT TAC C-3'	
qPCR	D1		5'-TGT GCT CAT GTG TTT AAA CTT-3'	
	D2		5'-CAA AAC TAT AGT TAC CAA TTT TG-3'	
	Crt76_CVMNK	FAM	5'-TGT GTA ATG AAT AAA ATT TTT GCT AA-3'	BHQ1
	Crt76_CVIET	JOE	5'-TGT GTA ATT GAA ACA ATT TTT GCT AA-3'	BHQ1
	Crt76_SVMNT	ROX	5'-AGT GTA ATG AAT ACA ATT TTT GCT AA-3'	BHQ2

Polymorphisms in the loci of interest of *pfmdr1* gene were identified by direct sequencing of PCR products, as described.[273] PCR amplifications and direct sequencing were performed on four different fragments of *pfmdr1* encompassing codons of interest 86 and 184 (fragment 1), codons 1034, 1042 and 1246 (fragment 2), codons 1034 and 1042 (fragment 3), and codon 1246 (fragment 4). Amplification on fragment 3 and fragment 4 were performed when PCR failed to amplify fragment 2 of *pfmdr1*. Nest 1 reaction mixture contained 5 µL of DNA, NH_4 reaction buffer, 2 mM $MgCl_2$, 1 mM of dNTPs, 200 nM of each primer, and 1 unit of BIOTAQ (Bioline, UK). The nest 2 reaction contained 1 µL of nest 1 product, NH_4 reaction buffer, 2 mM $MgCl_2$, 1 mM of dNTPs, 200 nM of each primer, and 1 unit of BIOTAQ (Bioline, UK). Primers and cycling conditions for each fragment are described in Table 5.2. PCR products of nest 2 amplifications were detected using 2% agarose gel electrophoresis and ethidium

bromide staining. PCR products were subsequently sequenced using BigDye Terminator v3.1 cycle sequencing kits and analysis on an ABI 3730 sequencer (Applied Biosystems). The sequences were analysed using Geneious software (Version 8.0.5, Biomatters, New Zealand).

Table 5.2. Primers and cycling conditions for *pfmdr1* PCR amplifications[273]

Gene	Primers	Sequence	PCR product size	PCR conditions
Pfmdr1 Fragment 1	Fwd1-FN1/1 Rev1-REV/C1	5'-AGGTTGAAAAAGAGTTGAAC-3' 5'-ATGACACCACAAACATAAAT-3'	578 bp	94°C 3 mins/30x (94°C 30 secs, 55°C 30 secs, 65°C 1 min)/ 65°C 5 mins
	Fwd2-MDR2/1 Rev2-NEWREV1	5'-ACAAAAAGAGTACCGCTGAAT-3' 5'-AAACGCAAGTAATACATAAAGTC-3'	534 bp	94°C 3 mins/30x (94°C 30 secs, 60°C 30 secs, 65°C 1 min)/ 65°C 5 mins
Pfmdr1 Fragment 2	Fwd1-MDRFR2F1 Rev1-MDRFR2R1	5'-GTGTATTTGCTGTAAGAGCT-3' 5'-GACATATTAAATAACATGGGTTC-3'	958 bp	94°C 3 mins/34x (94°C 30 secs, 55°C 1 min, 65°C 1 min)/ 65°C 5 mins
	Fwd2-MDRFR2F2 Rev2-MDR2FR2R2	5' CAGATGATGAAATGTTTAAAGATC-3' 5'-TAAATAACATGGGTTCTTGACT-3'	864 bp	94°C 3 mins/29x (94°C 30 secs, 60°C 30 secs, 65°C 1 min)/ 65°C 5 mins
Pfmdr1 Fragment 3	Fwd1-MDRF3N1 Rev1-MDRF3R1	5'-GCA TTT TAT AAT ATG CAT ACT G-3' 5'-GGA TTT CAT AAA GTC ATC AAC-3'		94°C 3 mins/30x (94°C 30 secs, 56°C 60 secs, 65°C 50 secs)/ 65°C 5 mins
	Fwd2-MDRF3N2 Rev2-MDRF3R1	5'-GGT TTA GAA GAT TAT TTC TGT A-3' 5'-GGA TTT CAT AAA GTC ATC AAC-3'	234 bp	Same as primary PCR above
Pfmdr1 Fragment 4	Fwd1-MDRF4N1 Rev1-MDRF4R1	5'-CAA ACC AAT CTG GAT CTG CAG-3' 5'-CAA TGT TGC ATC TTC TCT TCC-3'		94°C 3 mins/30x (94°C 30 secs, 55°C 60 secs, 65°C 40 secs)/ 65°C 5 mins
	Fwd2-MDRF4N2 Rev2-MDRF4R1	5'-GAT CTG CAG AAG ATT ATA CTG-3' 5'-CAA TGT TGC ATC TTC TCT TCC-3'	194 bp	Same as primary PCR above

Amplifications of *pfk13* propeller domain were performed by nested PCR amplification.[268] PCR master mix for nest 1 were performed in 25 µL reaction mixture containing 5 µL of DNA template, 0.25 µM of each primer and 1X HOT FirePol Master Mix (Solis Biodyne), with cycling conditions as follow: initial denaturation at 95 °C for 15 minutes, followed by 30 cycles of 95 °C for 30 seconds, 58 °C for 2 minutes, and 72 °C for 2 minutes, followed by final extension at 72 °C for 10 minutes. For nest 2 amplification, a 50 µL of reaction mixture was prepared containing 5 µL of nest 1 product, 0.25 µM of each primer and 1X HOT FirePol Master Mix (Solis Biodyne). Cycling conditions for nested PCR were as follows: initial denaturation at 95 °C for 15 minutes, followed by 40 cycles of 95 °C for 30 seconds, 60 °C for 1 minutes, and 72 °C for 1 minutes, followed by final extension at 72 °C for 10 minutes. Primer sets for both PCR assays are provided in Table 5.3.[268] Nested PCR products were then sequenced using BigDye Terminator v3.1 cycle sequencing kits and analysis on an ABI 3730 sequencer (Applied Biosystems). Polymorphisms were analysed using Geneious software (Version 8.0.5, Biomatters, New Zealand).

Table 5.3. Primers for *pfk13* gene amplifications[268]

Reaction	Primer	Sequence
1st	K13-1	5′-CGG AGT GAC CAA ATC TGG GA-3′
	K13-4	5′-GGG AAT CTG GTG GTA ACA GC-3′
2nd	K13-2	5′-GCC AAG CTG CCA TTC ATT TG -3
	K13-3	5′-GCC TTG TTG AAA GAA GCA GA -3′

5.2.3 Statistical analysis

Statistical analyses were performed using Stata IC (version 15, StataCorp, TX, USA). Binary variables were used to compare across pairs of categories. Odds ratios (ORs) with 95% confidence intervals (CIs) were estimated, and significance was determined using X^2 distribution.

5.3 Results

PCR detected 404 (10.8%) individuals with *P. falciparum* infections (additional *pfmdr1* genotyping detected 11 *P. falciparum* cases to the total numbers presented in Chapter 4), of which 65% were subpatent (Table 5.4.). Batubara, Langkat and South Nias each provided 81 (20.0%), 90 (22.3%) and 233 (57.7%) *P. falciparum* isolates, respectively.

Table 5.4. Prevalence of *P. falciparum* infection detected by microscopy and PCR

Pf* microscopy	Pf* PCR		Total
	Positive	Negative	
Batubara			
Positive	36	57	93
Negative	45	1132	1234
Sub-total	81	1189	1270
Langkat			
Positive	33	41	74
Negative	57	413	470
Sub-total	90	454	544
South Nias			
Positive	75	96	171
Negative	158	1588	1746
Sub-total	233	1684	1917
Total	**404**	**3327**	**3731**

*Pf=*P. falciparum*

5.3.1 Polymorphisms in *pfcrt* gene

Pfcrt genotyping at codons 72 to 76 was successful only in 183 (45.3%) samples. Low PCR success rate observed here may be due to high proportion of submicroscopic infections among our samples, in addition to lesser sensitivity of qPCR assay compared to the nested PCR assay used to identify *P. falciparum* infections.[353] For this analysis, mutant 76T was present in 91.8% (168 of 183, 95% CI 86.9-94.9) of samples. The *pfcrt*-SVMNT was confirmed to be the predominant haplotype as observed in 76.5% (140 of 183; 95% CI 69.9-82.1) of samples, presented either alone (68.6%) or mixed with other genotypes (31.4%). Parasites carrying SVMNT were also the most prevalent in all three sites with 85.7% (42 of 49; 95% CI 73.3-92.9) in Batubara, 84.6% (33 of 39; 95% CI 70.3-92.8) in Langkat, and 68.4% (65 of 95; 95% CI 58.5-76.9) in South Nias. The remaining haplotypes, calculated by combining isolates with single and mixed genotypes, were less common being detected at 34.9% (64/183; 95% CI 28.4%-42.1%) for wild-type CVMNK and 20.2% (37/183; 95% CI 15.0%-26.6%) for mutant-CVIET haplotypes

(Figure 5.1.). Although overall proportion of CVIET-parasites was low, but in South Nias this mutant genotype was relatively more common (34/95; 35.8%, OR 3.67, 95% CI 1.54-9.42, *P*=0.001) (Table 5.5.).

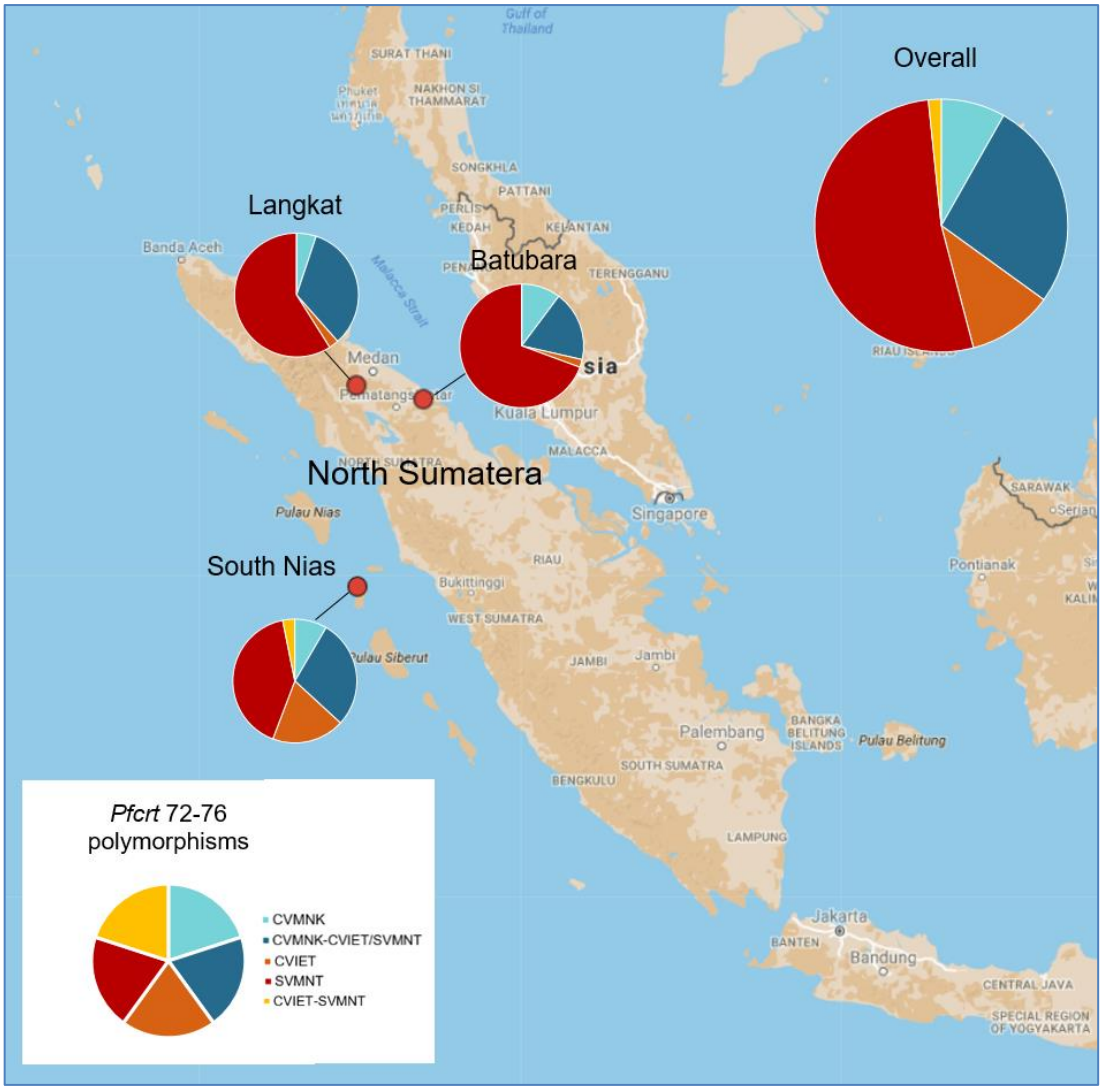


Figure 5.1. Proportion of *pfcrt* polymorphisms in three study sites in North Sumatera province (*n*=49 for Batubara, *n*=39 for Langkat, and *n*=95 for South Nias).

Table 5.5. Proportion of *pfcrt* polymorphisms according to sites

Pfcrt 72-76 haplotype	Batubara, *n* (%)		Langkat, *n* (%)		South Nias, *n* (%)		Overall, *n* (%)
	Patent	Subpatent	Patent	Subpatent	Patent	Subpatent	
CVMNK	2/49 (4.0)	3/49 (6.0)	1/39 (2.6)	1/39 (2.6)	3/95 (3.2)	5/95 (5.3)	15/183 (8.2)
CVIET	0/49 (0)	1/49 (2.0)	0/39 (0)	1/39 (2.6)	4/95 (10.3)	14/95 (14.7)	20/183 (10.9)
SVMNT	23/49 (46.9)	11/49 (22.4)	15/39 (38.5)	8/39 (20.5)	16/95 (16.8)	23/95 (24.2)	96/183 (52.5)
CVMNK-SVMNT	3/49 (6.1)	3/49 (6.0)	6/39 (15.4)	3/39 (7.7)	8/95 (3.2)	12/95 (12.6)	35/183 (19.1)
CVMNK-CVIET	0/49 (0)	1/49 (2.0)	0/39 (0)	3/39 (7.7)	0/95 (0)	4/95 (10.3)	8/183 (4.4)
CVIET-SVMNT	0/49 (0)	0/49 (0)	0/39 (0)	0/39 (0)	1/95 (1.1)	2/95 (2.1)	3/183 (1.6)
CVMNK-CVIET-SVMNT	1/49 (2.0)	1/49 (2.0)	1/39 (0)	0/39 (0)	1/95 (1.1)	2/95 (2.1)	6/183 (3.3)

5.3.2 Polymorphisms in *pfmdr1* gene

Analysis of polymorphisms in *pfmdr1* gene were successful in 267, 262, 72, 74 and 70 isolates for codons 86, 184, 1034, 1042 and 1246, respectively. Low success rates were seen for codons 1034, 1042 and 1246 genotyping and these were likely due to the difficulties to amplify the large fragment 2 of *pfmdr1*. But when amplifications were done separately for codon 1034 and 1042 in fragment 3, and 1246 in fragment 4, success rate was not improved. Low density parasites might have contributed to this poor success, and furthermore, higher success in fragment 1 amplification was likely due to better sensitivity of the PCR assay for fragment 1 amplification compared to that of other fragments. Sequences from the successful amplifications at these alleles of interest were then classified as wild-type (identical to reference 3D7 strain), mutant or mixed genotype. Prevalence of wild-type N86 differed according to study site, with the lowest at 38.9% (23 of 59; 95% CI 27.6-51.7) in Batubara, 57.6% (34 of 59; 95% CI 44.9-69.4) in Langkat, and 78.5% (117 of 149; 95% CI 71.3-84.4) in South Nias (Table 5.6.). Mutation to Tyrosine in allele 86 was the most prevalent mutation in all three sites (overall prevalence 34.8%, 95% CI 29.4-40.7), and in Batubara appeared in the majority of isolates (37 of 59; 62.7%, 95% CI 49.9-73.9). Rare mutations in allele 86 to phenylalanine (F) and to serine (S) were also observed (Figure 5.2.), each was identified twice (0.8%). Rare mutant 86F was previously described in two isolates from Swaziland[354], however we are not aware of any report of mutant 86S. In the locus of interest 184, prevalence of wild-type parasite carrying Y184 was the highest; 94.9% (56 of 59; 95% CI 86.1-98.3) in Batubara, 89.5% (51 of 57; 95% CI

78.9-95.1) in Langkat and 80.8% (118 of 146; 95% CI 73.7-86.4) in South Nias. Only very small proportion of samples harbored the mutant genotype 184F (39 of 262; 14.9%, 95% CI 11.1-19.7), (Figure 5.3.). Whereas for the remaining alleles of interest, only wild-type parasites were detected at codons 1034, 1042 and 1246.

Table 5.6. Proportion of polymorphisms in *pfmdr1* gene according to sites

Pfmdr1	Batubara, *n* (%)		Langkat, *n* (%)		South Nias, *n* (%)		Overall, *n* (%)
	Patent	Subpatent	Patent	Subpatent	Patent	Subpatent	
N86	4/59 (6.8)	18/59 (3.1)	4/59 (6.8)	27/59 (45.8)	31/149 (20.8)	86/149 (57.7)	170/267 (63.7)
N86Y	1/59 (1.7)	0/59 (0)	2/59 (3.4)	1/59 (1.7)	0/149 (0)	0/149 (0)	4/267 (1.5)
86Y	26/59 (44.1)	10/59 (16.9)	19/59 (32.2)	5/59 (8.5)	18/149 (12.1)	11/149 (7.4)	89/267 (33.3)
86S	0/59 (0)	0/59 (0)	1/59 (1.7)	0/59 (0)	1/149 (0.7)	0/149 (0)	2/267 (0.8)
86F	0/59 (0)	0/59 (0)	0/59 (0)	0/59 (0)	0/149 (0)	2/149 (1.3)	2/267 (0.8)
Y184	29/59 (49.2)	26/59 (44.1)	23/57 (40.4)	28/57 (49.1)	40/146 (27.4)	77/146 (52.7)	223/262 (85.1)
Y184F	1/59 (1.7)	0/59 (0)	0/57 (0)	0/57 (0)	0/146 (0)	1/146 (0.001)	2/262 (0.8)
184F	1/59 (1.7)	2/59 (3.4)	2/57 (3.5)	4/57 (7.0)	8/146 (5.5)	20/146 (13.7)	37/262 (14.1)

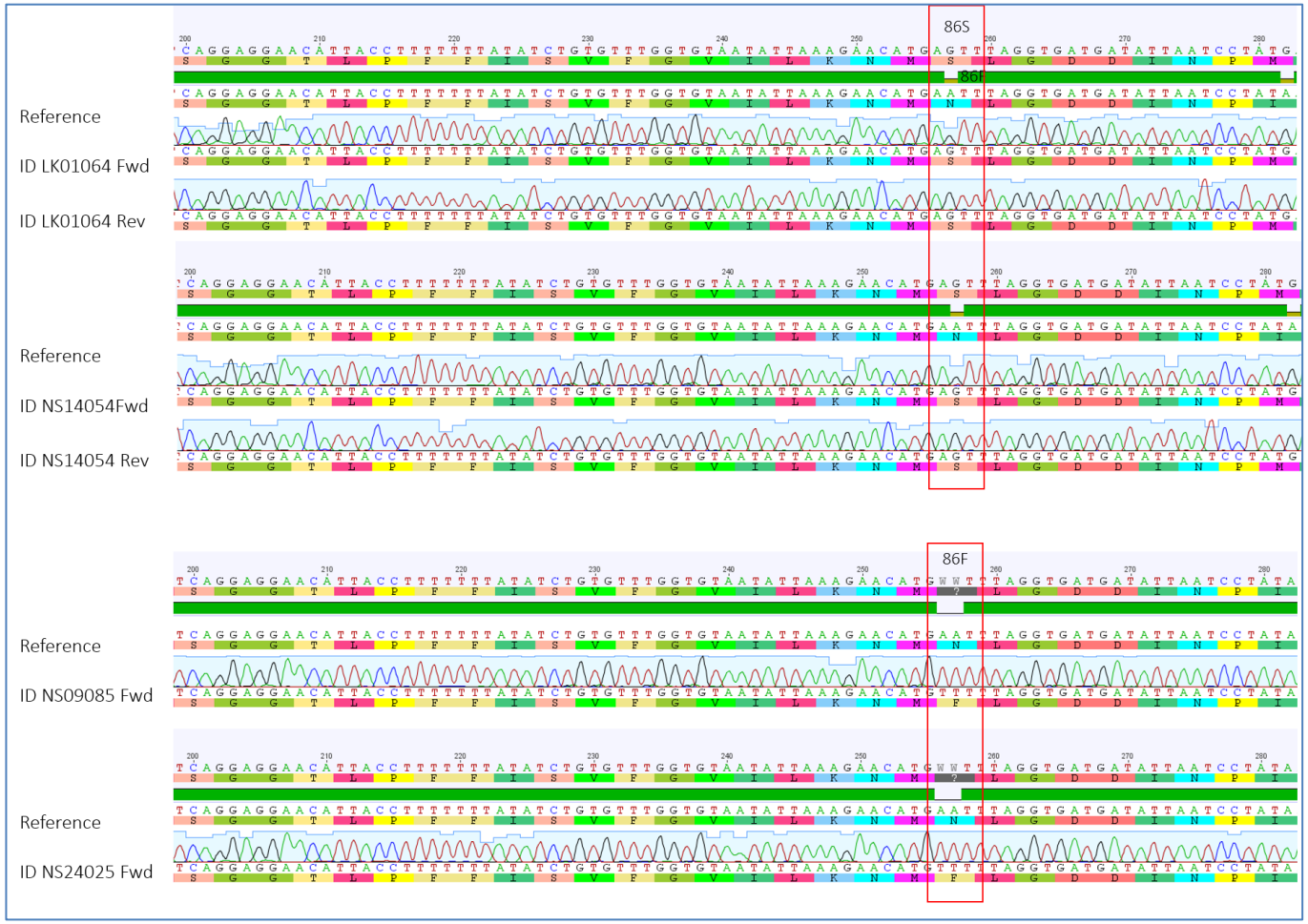


Figure 5.2. Mutation at *pfmdr1* 86S and 86F as observed in each two isolates

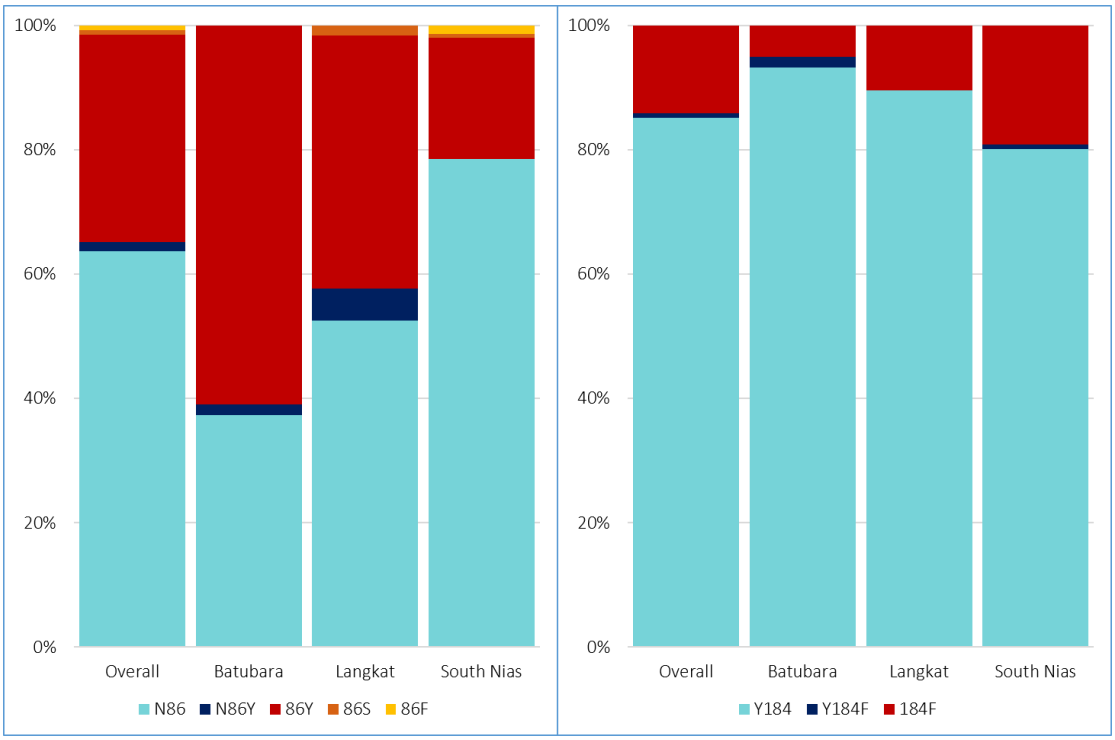


Figure 5.3. Polymorphisms at alleles 86 and 184 of *pfmdr1* according to study sites (*n*=267 for codon 86, and *n*=262 for codon 184).

To determine the association between the polymorphisms in *pfmdr1* with drug response, we then assembled the genotypes of alleles 86, 184 and 1246. Haplotype NFD at these positions has been associated with reduced sensitivity to AL, while on the other hand, amodiaquine select parasites carrying the opposite haplotype, YYY.[273] Considering most isolates failed to genotype at codon 1246, the haplotype analysis was restricted to isolates successfully genotyped at both codons 86 and 184. We also included samples with mixed alleles, assuming that one haplotype of interest occured. Based on this criteria, we observed that haplotype-YY was most prevalent in Batubara (OR 4.6, 95% CI 2.4-8.9, *P*<0.001) compared to other regencies, haplotype-YY and haplotype-NY were equally common in Langkat, and haplotype-NY was the most common in South Nias (Table 5.7.). Both in Batubara (OR 31.4, 95% CI 8.3-169.4, *P*<0.001) and Langkat (OR 8.7, 95% CI 2.8-31.5, *P*<0.001), parasites were more likely to carry the YY-haplotype than the NF-haplotype, as expected. Whilst, in South Nias both haplotypes YY and NF were equally distributed (Figure 5.4.). Interestingly, although different haplotypes were prevalent at different study sites, we observed that parasites carrying YY alleles was significantly more prevalent as patent infection than any other alleles at all sites (Batubara, OR 9.7, 95% CI 2.5-41.2, P<0.0001; Langkat, OR 15.0, 95% CI 3.6-65.4, P<0.0001; South Nias, OR 4.9, 95% CI 1.9-13.4, P<0.0002).

Table 5.7. Proportion of haplotypes 86-184 of *pfmdr1* according to sites

Pfmdr1 86-184 haplotype	Batubara, *n* (%)		Langkat, *n* (%)		South Nias, *n* (%)		Overall, *n* (%)
	Patent	Subpatent	Patent	Subpatent	Patent	Subpatent	
NY	3/59 (5.1)	16/59 (27.1)	5/57 (8.8)	23/57 (40.4)	22/145 (15.2)	66/145 (45.5)	135/261 (51.7)
NF	1/59 (1.7)	2/59 (3.4)	1/57 (1.8)	4/57 (7.0)	6/145 (4.1)	20/145 (13.8)	34/261 (13.0)
YY	27/59 (45.8)	10/59 (16.9)	20/57 (35.1)	6/57 (10.5)	18/145 (12.4)	10/145 (6.9)	91/261 (34.9)
YF	1/59 (1.7)	0/59 (0)	0/57 (0)	0/57 (0)	0/145 (0)	1/145 (0.7)	2/261 (0.8)

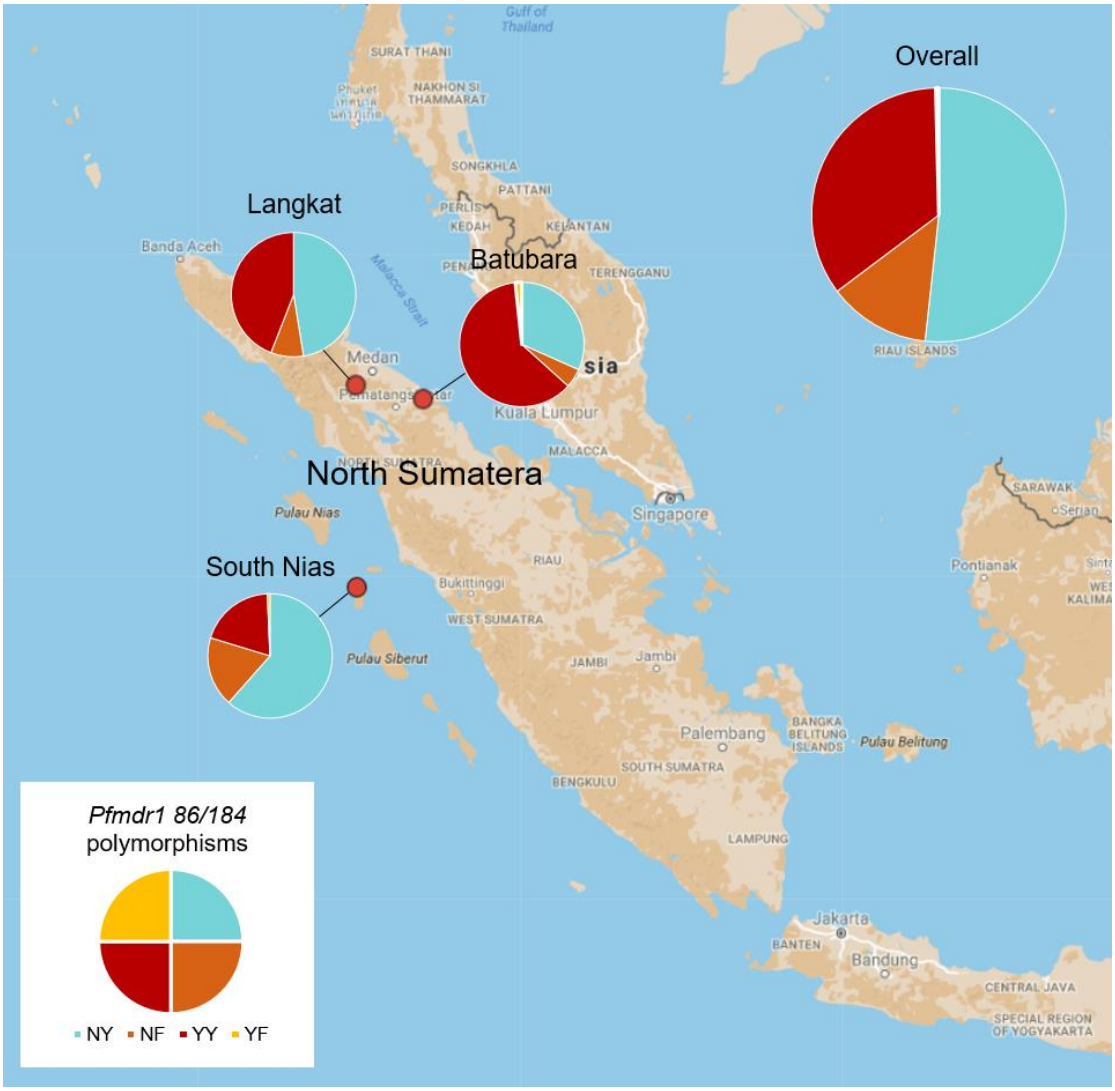


Figure 5.4. Proportion of each haplotype at codons 86-184 of *pfmdr1* in three study sites in North Sumatera province (*n*=59 for Batubara, *n*=57 for Langkat, and *n*=145 for South Nias).

5.3.3 Polymorphisms in *pfk13*

Genotyping for the *pfk13* gene was successful in 232 (57.4%) isolates. As previously described before, submicroscopic infections may have contributed to this low rate of success. Of these, 95.7% (222/232; 95% CI 92.2-97.6) harbored wild-type parasites, and 4.3% (10/232, 95% CI 2.4-7.8) carried mutations (Table 5.8., Figure 5.5.). As this is the first study to show some proportion of *pfk13* mutations in the parasite population in western Indonesia, we did extra measures before providing the evidence of these mutations among our samples (summarized in Table 5.9.). Ten isolates contained 24 non-synonymous polymorphisms. Of those, only one isolate carried single mutation, two had one mutation mixed with wild-type parasite, and the remaining had > 1 non-synonymous mutation with or without the presence of wild-type parasite. Mutant T474A, the most frequently mutant observed, was occurred in 7 isolates across three different sites, all presented mixed with wild-type parasite. Mutation at codon 535, a change from threonine to alanine, was observed in 2 isolates from Batubara. Whereas, the remaining mutations were observed only once. The C580Y mutation, currently the most prevalent K13 genotype in western Cambodian parasites and is associated with delay clearance, was only seen in 1 patient from South Nias (Figure 5.5.). While other mutations previously reported to be associated with slow parasite clearance was not seen among our samples. There was no difference in the prevalence of K13 mutation between study sites (P>0.05).

Table 5.8. Proportion of non-synonymous mutations in *P. falciparum* kelch13 propeller-domain

Pfk13	Batubara, *n* (%)		Langkat, *n* (%)		South Nias, *n* (%)		Overall, *n* (%)
	Patent	Subpatent	Patent	Subpatent	Patent	Subpatent	
Wild-type	30/66 (45.5)	32/66 (48.5)	27/60 (45.0)	29/60 (48.3)	34/106 (32.1)	70/106 (66.0)	222/232 (95.7)
Mutant	1/66 (1.5)	3/66 (4.5)	1/60 (1.7)	3/60 (5.0)	0/106 (0)	2/106 (1.9)	16/232 (4.3)

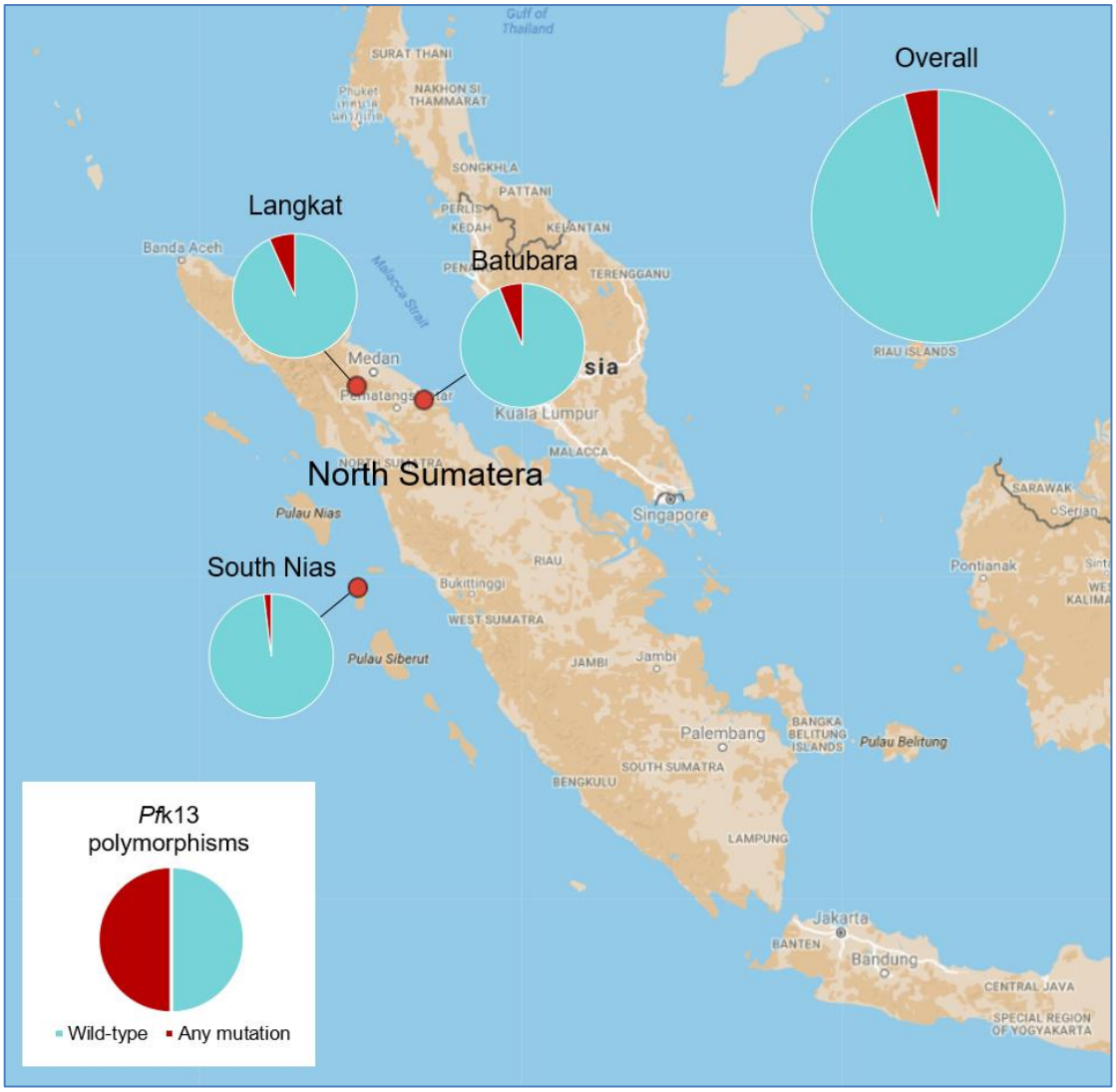


Figure 5.5. Proportion of *P. falciparum* kelch13 mutations in three study sites in North Sumatera province (*n*=66 for Batubara, *n*=60 for Langkat, and *n*=106 for South Nias).

Table 5.9. Non-synonymous single-nucleotide polymorphisms in the *pfk13* propeller domain

Codon	Reference sequence	AA	Nucleotide substitution	AA change	Frequency, *n* (%)	Presence of wild-type allele	Evidence**
Y456H	Y	Tyr	t1366c	H	1 (0.4)	+	+
E461G	E	Glu	a1382g	G	1 (0.4)	+	+
T474A	T	Thr	a1420g	A	7 (3.0)	+	+
S521P	S	Ser	t1561c	P	1 (0.4)	-	+
N523S	N	Asn	a1568g	S	1 (0.4)	-	+ / -
T535A	T	Thr	a1603g	A	2 (0.9)	-	+ / -
Y541H	Y	Tyr	t1621c	H	1 (0.4)	+	+
C542R	C	Cys	t1624c	R	1 (0.4)	-	+
V568A	V	Val	t1703c	A	1 (0.4)	+	+ / -
C580Y*	C	Cys	g1739a	Y	1 (0.4)	+	+
T593A	T	Thr	a1777g	A	1 (0.4)	-	+ / -
N609S	N	Asn	a1826g	S	1 (0.4)	-	+
Y616H	T	Thr	t1846c	H	1 (0.4)	-	+
F628L	F	Phe	t1882c	L	1 (0.4)	+	+
N642S	N	Asn	a1925g	S	1 (0.4)	-	+
P655S	P	Pro	c1963t	S	1 (0.4)	-	+

*Mutation in C580Y, associated with delay in parasite clearance in Greater Mekong parasites
**+/- denotes weak evidence, on a single strand only, or not confirmed by subsequent repeat PCR

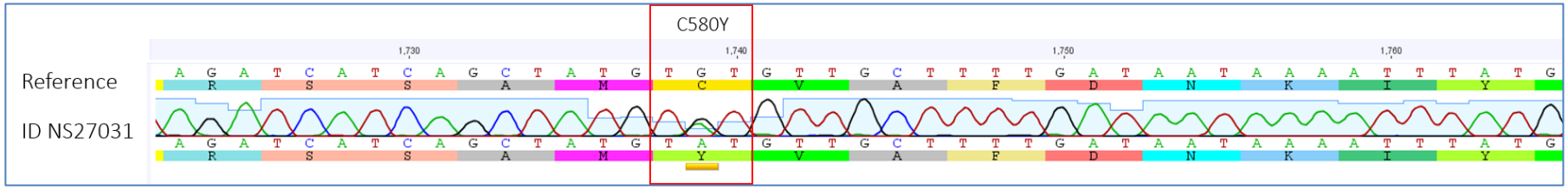


Figure 5.6. Mutation at C580Y, a change from cysteine to leucine, in one sample from South Nias. Despite repeat experiments, this was only observed in one strand.

5.3.4 Associations between the *pfcrt*, *pfmdr1* and *pfk13* polymorphisms among Sumatera *P. falciparum* isolates

The relationship between putative molecular markers, *pfcrt* and *pfmdr1*, has been previously characterized where mutations in these genes were linked to a variety of degree of resistance to chloroquine and amodiaquine.[181, 355] In African settings, the *pfcrt* 76T mutation appeared exclusively in the background of *pfmdr1* 86Y mutation.[356] In Southeast Asia, CVIET-form appeared in a wild-type or mutant allele of *pfmdr1*.[357] Combination of mutations in these two genes confer different levels of sensitivity to drugs.[181, 355] Therefore, we further evaluated the association between polymorphisms in the tested genes in this study. Analysis of the *pfcrt* gene at codons 72 to 76 revealed that SVMNT variant was predominant across the three study sites. This haplotype demonstrated a strong association with the YY-haplotype of *pfmdr1* (X^2=33.36, 1 degree of freedom; P<0.0001). This suggests that the SVMNT haplotype of *pfcrt* and the YY haplotype of *pfmdr1* are in linkage disequilibrium in these parasite populations. *Pfk13* mutant alleles also appeared in the background of SVMNT parasites, being detected 3 of 3 (100%) of successfully evaluated isolates. However, these mutants appeared in both *pfmdr1* background between parasites carrying YY haplotype (4 of 7, 57.1%) and NY haplotype (3 of 7, 42.9%). Nevertheless, these numbers are too small to give conclusive evidence.

5.4 Discussion

This study provides genetic characterisation of *P. falciparum* parasites from north-western Indonesia. We evaluated the frequency of polymorphisms in the *pfcrt*, *pfmdr1* and *pfk13*, the drug resistance markers for chloroquine, amodiaquine, mefloquine, lumefantrine and artemisinin.[181, 268, 269, 273, 349, 355] In North Sumatera, the prevalence of mutant *pfcrt* 76T, the resistance marker for aminoquinoline, were at 90% in 2004 before the initiation of ASAQ.[108, 109] Recently, in our study we show that this prevalence has been maintained at 91%, with a similar observation in different parts of Indonesia.[109, 282] Although the discontinuation of chloroquine is often accompanied by the return of wild-type *pfcrt* 76K[358], continuous selection to the mutant *pfcrt* 76T has also been reported when chloroquine is replaced by ACT-containing amodiaquine.[197, 359] Many studies have shown a strong selective pressure to mutant *pfcrt* with the use of ASAQ, while AL exerts selection favouring the wild-type genotype.[197, 273] Other studies have also shown a better fitness advantage of SVMNT haplotype compared to the CVIET form, where this former haplotype persists even after chloroquine is withdrawn[286, 360] and CVIET-variant is replaced by the wild-type haplotype with the abandonement of chloroquine.[358, 361] Moreover, haplotype SVMNT of codons 72-76 *pfcrt* alone has shown to be sufficient to confer high-level resistance to amodiaquine in *in vitro* studies.[181, 355] The *pfcrt* SVMNT was the dominant haplotype (76.5%) observed in our settings, and our findings suggest that reduced susceptibility of parasites to ASAQ previously shown in *in vivo* studies across Indonesia might be explained by the moderate to high proportion of mutant SVMNT in the country.[279, 282]

Resistance to chloroquine and amodiaquine is also known to be mediated by variation at the *pfmdr1* locus, encoding the PgH1. Alleles 86Y, 184Y and 1246Y are selected following treatment with ASAQ.[273] Unlike to parasites carrying SVMNT, African/Southeast Asian parasites harboring CVIET-variant of *pfcrt* require these *pfmdr1* mutations to confer higher grade of amodiaquine resistance.[273, 349, 355] The prevalence of mutant N86Y in our study was different between sites. In 2003, all parasites tested in Nias were reported to harbour this mutant genotype before the prevalence decreased to 31.4% in 2005[107, 109] and recently to as low as 19.5% in our study. However, the prevalences in Batubara (62.7%) and Langkat (45.8%) were still relatively moderate. The prevalence of wild-type allele Y184 is higher than that of N86 and estimated as above 80% in all three sites contributing in some proportion of wild-type *pfmdr1* 86N/184Y in each sites. However, *in vitro* studies suggest that the impact of

polymorphisms in allele 184 to drug sensitivity are small and very dependent on the status of allele 86.[355] In North Sumatera, mutant *pfmdr1* 86Y/184Y was the most prevalent in Batubara (62.7%) and slightly more predominant than wild-type halotype in Langkat (45.6%). This YY haplotype greatly reduced susceptibility to amodiaquine *in vivo*, whereas AL provides better sensitivity and selects the wild-type parasite NF. This selection has been evidently shown in studies where CVIET parasites are prevalent.[273] Nevertheless, a recent gene-editing study showed parasites with the background line of SVMNT modified with *pfmdr1* YY genotype are also associated with increased sensitivity to lumefantrine and mefloquine.[355]

Selection by piperaquine on *pfmdr1* is still unclear. Studies have shown conflicting results on the selective pressure by DP, with few studies in Uganda showing similar selection as in aminoquinoline resistance[275, 362], but distinctive selection in some other studies in western Africa.[363, 364] At the time of our sample collection, DP had been deployed for nearly 3 years in western Indonesia. However, due to poor access to treatment in our study sites, we cannot say for certain whether DP had already been used extensively in these region and had selected certain genotype of the *pfmdr1* gene. Our findings showed most isolates from Batubara and Langkat harboured *pfmdr1* YY parasites, and parasites in South Nias carried *pfmdr1* NY suggesting that *pfmdr1* polymorphisms in these parasites were the results of previous drug pressures from chloroquine and amodiaquine. These findings nevertheless required further investigation. Prospective data from the *in vivo* study will provide a better opportunity to study the association between phenotypic response to DP and parasite genotype.

Our findings also permit some comparison with recent important findings on the rise of artemisinin resistance in the Greater Mekong sub-region. To our knowledge, this is the first study from western Indonesia to provide evidence of K13 polymorphisms among local parasites. Resistance to artemisinin is known to be mediated by mutation in the *pfk13* propeller domain, with two independent emergence events having been identified in the region of Cambodia, Vietnam and Laos, and in the region of Thailand, Myanmar, and China.[301] Several nonsynonymous mutations; F466I, N458Y, N537D, R539T, I543T, P553L, P574L and C580Y, have been associated with day 3 positivity by microscopy and are considered indicators of clinical resistance to artemisinin.[302] In this study, wild-type K13 is still prevalent in over 90% of samples. Only a handful of K13 polymorphisms were documented. Among those, evidence was found in one sample of C580Y, the dominant haplotype in western Cambodia and Myanmar-Thailand[255, 302], which in this study appeared as subpatent infection. Parasites

carrying the wild-type allele were also present in this isolate (Figure 5.6.), and repeat PCR failed to confirm presence of the mutant allele (Table 5.9.). However, the presence of mutant T474A in 7 (3%) individuals raises interests on the possible phenotypic impact. Changes in codon 474 in propeller domain of K13 has previously been observed in small numbers of parasites from western and northeastern Cambodia[268], and later in Vietnam.[302] However changes were seen from threonine to isoleucine without any impact on clinical resistance to artemisinin.[268, 302] Nevertheless, it is still necessary to evaluate the growing trend of K13 polymorphisms in this region and whether other genetic changes will facilitate this selection in the future.

This study provides important evidence on genetic characterisation of *P. falciparum* parasites from North Sumatera. We have shown that *pfcrt* SVMNT is still highly circulating in our three study sites, while prevalence on *pfmdr1* 86Y were high in Batubara and Langkat, but has decreased markedly in South Nias, compared to earlier studies. We also have provided preliminary evidence of potentially important polymorphisms in *pfk13* gene region encoding the kelch13 propeller domain among our samples, although we cannot yet establish the association between our K13 findings with artemisinin resistance. We found no evidence of the spread of artemisinin-resistant parasites from neighboring Cambodia and Myanmar, as this phenotype only occurs on the *pfcrt* background of the CVIET in the Mekong[357], whereas the SVMNT background dominates in Sumatera (Table 5.5.) Nonetheless, our study has some limitations. As described before, we observed low success rate of PCR to genotype our samples. This may be due to low density of parasites and different levels of sensitivity of our PCR methods. We also did not perform the *pfmdr1* and *pfplasmepsin2* copy numbers measurements, which are the molecular markers for mefloquine and piperaquine resistance[199, 295, 351] and would be an important complement to our baseline genetic data. Lastly, we performed numbers of exploratory comparisons tests, which increased the chances of obtaining type I errors. Despite this, we decided not to use Bonferroni correction or other *post hoc* methods because in this exploratory study we want to generate new hypothesis for future testing which can be designed with adequate sample size.

Our current findings have important implications. The data can be used to guide future therapeutic efficacy studies and to recommend antimalarial policy in the region. The polymorphisms in the *pfcrt* and *pfmdr1* observed in this study are consistent with previous studies showing association with decreased susceptibility to amodiaquine, but increased sensitivity to lumefantrine component.[273, 355, 359] Although DP is not yet failing in this corner of

Southeast Asia, *in vivo* studies to evaluate the efficacy of this current treatment in comparison with AL or AS-MQ would provide comprehensive information for alternative treatment and improved understanding on local parasites. More importantly, if either of the regimen proves to provide better efficacy, switching to this drug, or alternating use with DP, will accelerate the goal to eliminate *P. falciparum* in this regions and to reach the status of malaria free.

Chapter 6

RESULTS 3

6 RESULTS

The efficacy of dihydroartemisinin-piperaquine versus artemether-lumefantrine against uncomplicated *Plasmodium falciparum* infection

6.1 Introduction

Artemisinin combination therapy (ACT) is the recommended first-line therapy by WHO for falciparum malaria since 2005. It only took few years for ACT to replace the previous mainstays of treatment namely chloroquine and sulfadoxine-pyrimethamine and be deployed as first-line treatment in most malaria endemic countries. Artemisinin is a very potent component and is combined with a partner drug to prevent development of resistance.[139] However, artemisinin is not entirely a new drug. It has been used as monotherapy to treat malaria for over 30 years in Southeast Asia.[123] Similar to previous antimalarials, decreased sensitivity to this component was also soon observed.[252, 253] Today, artemisinin-resistant parasites have been well documented but are confined to the Greater Mekong subregion.[255, 302]

Low sensitivity to artemisinin is now commonly observed in parts of Southeast Asia, however treatment failures are still uncommon unless artemisinin is combined with ineffective partner drugs.[255] Nevertheless, studies have shown that failures of artemisinin to clear parasites may lead to increased pressure to the partner drugs which subsequently cause selection of resistant-parasites. Thereafter, resistance to the partner drug would emerge.[118, 365] In addition, longer half-life provided by the partner drug also leaves this component at subtherapeutic level for longer period with a chance to develop and spread resistance.

Indonesia is endemic for malaria, but endemicity differs between areas. Western part of the country is subject to low transmission risk with *P. falciparum* and *P. vivax* co-endemic together.[88] Artesunate-amodiaquine (ASAQ) was used as the first-line antimalarial from 2004

before treatment failures led to its replacement by dihydroartemisinin-piperaquine (DP) in 2012.[128] Therapeutic efficacy studies on DP have shown high efficacy for both *P. falciparum* and *P. vivax* infections with excellent post-treatment prophylaxis effect resulting in delay of time to reinfection, greater time to haematological recovery and reduced chance for transmission.[176, 193, 220] However, with ACTs failing in Cambodia and Thailand, regular monitoring is essential to evaluate the efficacy of ACT in this region. Therefore, the aim of this study is to evaluate the efficacy of two ACTs, DP and artemether-lumefantrine (AL) in Indonesia and to define the extent of artemisinin resistance.

6.2 Methods

6.2.1 Study sites and design

We conducted a prospective, open-label, randomised comparison of DP and AL for the treatment of uncomplicated falciparum malaria. The study was designed using the 2009 World Health Organization (WHO) therapeutic efficacy protocol[172], with modification to include mixed infections and parasite density > 250 parasite/µL. The study was conducted in Batubara regency, Langkat regency and South Nias regency in North Sumatera province, western Indonesia between January and June 2015 (see Chapter 3).

The protocol was approved by the Ethics committee of the London School of Hygiene and Tropical Medicine (London, United Kingdom; 8504-01), and the Ethics committee of University of Sumatera Utara (Medan, Indonesia; 401/KOMET/FKUSU/2014). Written informed consent (Appendix 11 to 14) was obtained from adult patients and parents or guardians of enrolled children. The trial was registered with clinicaltrials.gov, number NCT02325180.

6.2.2 Patients

Patients were eligible if they were aged > 6 months, had acute symptomatic *P. falciparum* malaria confirmed by microscopy, parasitaemia > 250 parasite/µL, and temperature $\geq$ 37.5 °C or fever during the preceding 48 hours. Patients were excluded from the study if they were pregnant or lactating women, had haemoglobin < 7 g/dL, use of antimalarial in the past 2 weeks and appeared with danger signs or signs of severe malaria.

6.2.3 Study procedures

After enrolment, patients were randomly assigned to receive either DP or AL. Randomisation lists were computer-generated in blocks of 20, and treatment allocation was printed on numbered case record forms that was only seen after patients were enrolled. We recorded demographic information, detailed clinical symptoms, history of antimalarial use, and physical examination findings in the case record forms (Appendix 5). Finger prick blood

sampling was performed for microscopy examination and blood was spotted on filter paper (Whatman 3MM) for polymerase-chain reaction (PCR) genotyping.

Giemsa-stained thick blood films were assessed to determine parasite density against 200 white blood cells, and was calculated with the assumption of a white cell count of 8000 /µL. Blood smear is considered as negative after examination of 100 high power fields. Thin films were used to identify malaria species. All slides were single read by a certified microscopist.

Patients were examined every 24 hours until they became aparasitaemic, and they were monitored at day 7 and then weekly until day 42. At each appointment, we assessed clinical symptoms and performed physical examination including axillary temperature measurement. We also obtained both thick and thin blood smears and blood spots on filter papers. We measured haemoglobin levels at enrolment and on day 28 using a portable photometer (HemoCue® 301). For the full clinical protocol see Chapter 3, Appendices 1 to 12.

Patients with recurrent *P. falciparum* infection were classified as recrudescence or reinfection by *msp1*, *msp2* and *glurp* genotyping of samples collected at enrolment and day of failure. Reinfection is defined by a subsequent occurring parasitaemia in which all the alleles in parasites from the post-treatment sample are different from those in the admission sample, for one or more loci tested. While recrudescence is defined as at least one allele at each locus being common to both paired samples.[323] Nested polymerase chain reaction (PCR) reactions on *msp1* block (K1, MAD20 and RO33), *msp2* (FC27 and 3D7/IC) and *glurp* were performed using following previously published primers and cycling conditions.[366] Nest 1 amplifications were performed in 25 µL reaction mixture containing 5 µL DNA, NH_4 buffer, 4 mM $MgCl_2$, 1 mM deoxynucleotides (dNTPs), 0.2 µM forward primer, 0.2 µM reverse primer, and 1 unit of BIOTAQ (Bioline, UK). Cycling conditions for nest 1 amplification were: denaturation at 95 °C for 5 minutes, 25 cycles of 94 °C for 1 minute, 58 °C for 2 minutes and 72 °C for 2 minutes, followed by final extension of 5 minutes at 72 °C. Nest 2 amplifications were performed in 25 µL reaction mixture containing 1 µL of nest 1 product, NH_4 buffer, 4 mM $MgCl_2$, 1 mM dNTPs, 0.2 µM forward primer, 0.2 µM reverse primer, and 1 unit of BIOTAQ (Bioline, UK). Cycling conditions for nest 2 amplifications of *msp1* and *msp*2 were: denaturation at 95 °C for 5 minutes, 30 cycles of 94 °C for 1 minute, 58 °C for 2 minutes and 61°C for 2 minutes, followed by final extension of 5 minutes at 72 °C. Cycling conditions for *glurp* amplification were similar to nest 1 amplification. Ten µL of nest 2 products were analysed by electrophoresis in 2%

agarose gel in 0.5% TBE buffer stained with ethidium bromide. DNA was visualized using ultraviolet transillumination.

Table 6.1. Primers sequences for *msp1*, *msp2* and *glurp* genotyping[366]

Gene	Reaction	Primer	Sequence
msp1	Nest 1	M1-OF	5'-CTAGAAGCTTTAGAAGATGCAGTATTG-3'
	Nest 1	M1-OR	5'-CTTAAATAGTATTCTAATTCAAGTGGATCA-3'
	Nest 2-MAD20 family specific	M1-KF	5'-AAATGAAGAAGAAATTACTACAAAAGGTGC-3'
	Nest 2-MAD20 family specific	M1-KR	5'-GCTTGCATCAGCTGGAGGGCTTGCACCAGA-3'
	Nest 2-K1 family specific	M1-MF	5'-AAATGAAGGAACAAGTGGAACAGCTGTTAC-3'
	Nest 2-K1 family specific	M1-MR	5'-ATCTGAAGGATTTGTACGTCTTGAATTACC-3'
	Nest 1-RO33 family specific	M1-RF	5'-TAAAGGATGGAGCAAATACTCAAGTTGTTG-3'
	Nest 1-RO33 family specific	M1-RR	5'-CATCTGAAGGATTTGCAGCACCTGGAGATC-3'
msp2	Nest 1	M2-OF	5'-ATGAAGGTAATTAAAACATTGTCTATTATA-3'
	Nest 1	M2-OR	5'-CTTTGTTACCATCGGTACATTCTT-3'
	Nest 2-FC27 family specific	M2-FCF	5'-AATACTAAFAFTFTAFFTFCARATGCTCCA-3'
	Nest 2-FC27 family specific	M2-FCR	5'-TTTTATTTGGTGCATTGCCAGAACTTGAAC-3'
	Nest 2-3D7/IC family specific	M2-ICF	5'-AGAAGTATGGCAGAAAGTAAKCCTYCTACT-3'
	Nest 2-3D7/IC family specific	M2-ICR	5'-GATTGTAATTCGGGGGATTCAGTTTGTTCG-3'
glurp	Nest 1	G-OF	5'-TGAATTTGAAGATGTTCACACTGAAC-3'
	Nest 1 and 2	G-OR	5'-GTGGAATTGCTTTTTCTTCAACACTAA-3'
	Nest 2	G-NF	5'-TGTTCACACTGAACAATTAGATTTAGATCA-3'

6.2.4 Treatment

DP (Duo-Cotecxin, Holley Pharmaceutical, China; containing 40 mg dihydroartemisinin and 320 mg piperaquine) was given at a dose of 2.25 mg/kg/dose for dihydroartemisinin and 18 mg/kg/dose for piperaquine, rounded up to the nearest half tablet. DP was administered at enrolment, and after 24 hour and 48 hour. AL (Coartem, Novartis Pharma, China) was also dispensed according to weight, with half a tablet consisting of 20 mg artemether and 120 mg lumefantrine given at every 5 kg body weight. AL was administered at enrolment, hour 8, hour 24, hour 36, hour 48 and hour 60. Administration of all antimalarial drugs were supervised by a study staff. AL was given with drinking water on a full stomach, and biscuits and milk were provided for participants with an empty stomach. While DP was given in an empty stomach, and patients were instructed to fast for 2 hours after drug administration. As recommended in the Indonesian national guideline, additional single dose of primaquine at 0.75 mg/kg was given for *P. falciparum* infection and 14-day treatment of 0.25 mg/kg primaquine for *P. vivax* was also administered unsupervised on the final day of follow-up. G6PD testing was not performed prior to administration of primaquine.[128]

If vomiting occurred within 30 minutes, administration of a full dose was repeated. If vomiting happened in between 30 to 60 minutes, a half dose was administered. Recurrent

vomiting removed patients from the study. Patients failing therapy with recurrent *P. falciparum* was treated with quinine (10 mg of salt/kg given 3 times a day for 7 days) and doxycycline (100 mg twice a day for 7 days) if they were not children aged < 8 years old and not pregnant. Patient who developed infection with other *Plasmodium* infection during the study was re-treated according to the national guidelines.[128]

6.2.5 End points

The primary outcome was the efficacy of DP and AL for *P. falciparum* infection within 42 days after administration of the treatment, corrected by PCR genotyping. The secondary outcomes were parasite clearance times and proportion of patients with parasitaemia detected at 72 hours by microscopy.

6.2.6 Statistical analysis

Data were double-entered and analysed using Stata IC (version 15, StataCorp, TX, USA). We conducted analysis using a modified intention to treat method. Those who were lost to follow-up were considered to be censored, and withdrawn patients were considered as having treatment failure. Those whom we were not able to distinguish between recrudescence and reinfection due to failure to amplify *pfmsp1*, *pfmsp2* and *glurp* were regarded as not being treatment failure.

Normally distributed and non-normally distributed data were analysed using student t-test and rank-sum test. Cox-regression analysis was used to predict risk factor for treatment failures. Efficacy endpoints were determined by calculating the cumulative risk of recrudescence with *P. falciparum* at day 42 assessed with the use of Kaplan Meier survival analysis and Mantel-Haenszel log-rank test. *P* values of $\leq$ 0.05 were considered statistically significant.

6.3 Results

Of the 3731 screened participants, 302 met the selection criteria and were randomly assigned to receive DP or AL (Figure 6.1.). The number of patients in Batubara regency, Langkat regency and South Nias regency were each 36 (11.9%), 43 (14.2%), and 223 (73.8%), respectively. Of the 302 enrolled patients, 263 had *P. falciparum* infection and 39 had mixed *P. falciparum* and *P. vivax* infections identified by microscopy. Protocol violation [7] was observed within 72 hours in 9 (5.9%) and 3 (2.0%) patients in DP and AL groups, and patients were excluded from further analysis. Baseline characteristics were similar between treatment groups (Table 6.2.) Fifteen patients were lost to follow-up and considered as not being treatment failure and 8 patients withdrew from the trial and regarded as treatment failure. Overall, follow-up to day 42 or to the day of treatment failure was achieved in 92.4% (133/144) of patients who received DP and 91.1% (133/146) of those who received AL.

[7] Protocol violation is established when a patient do not complete the 3-day course treatment.

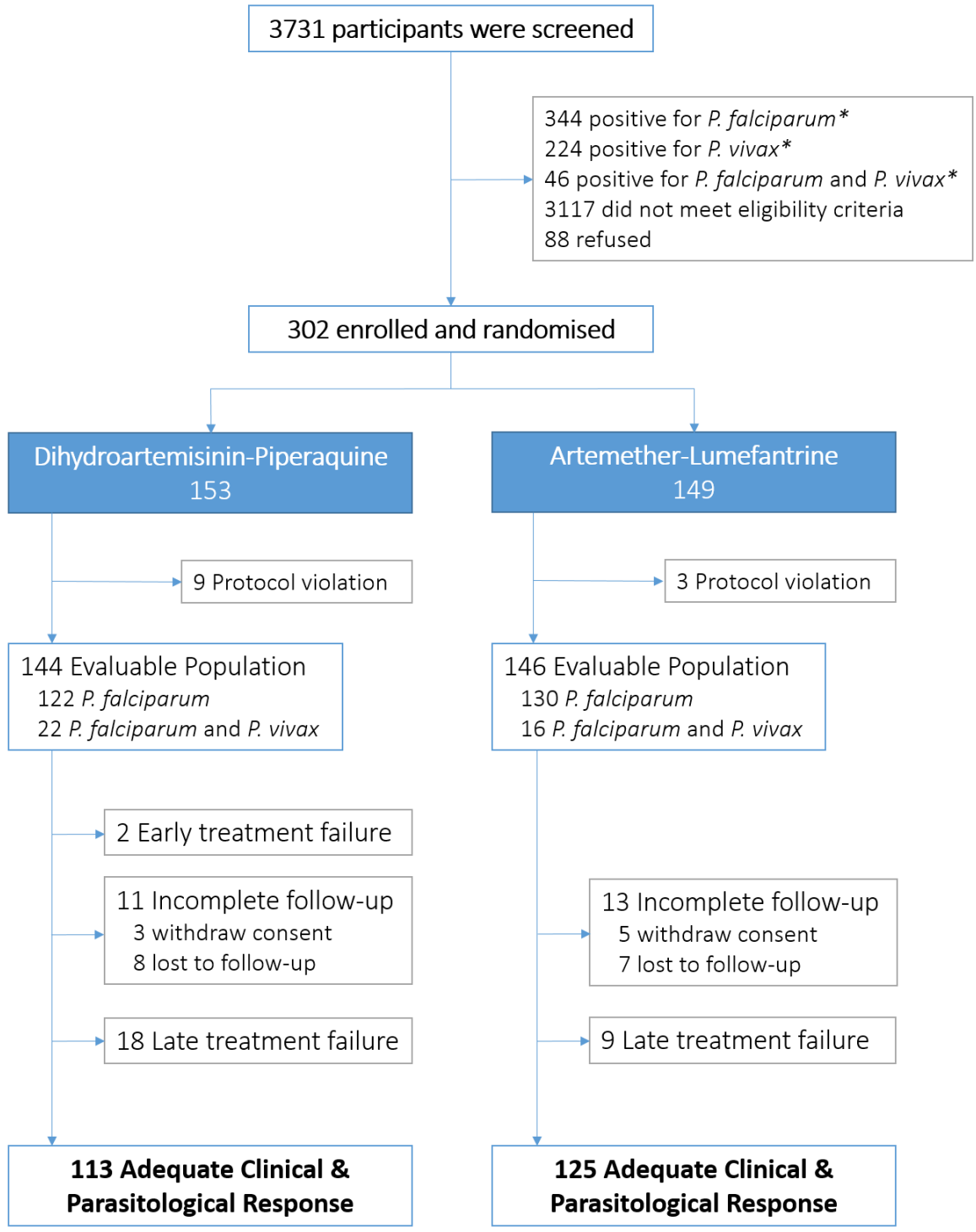


Figure 6.1. Profile of a study of dihydroartemisinin-piperaquine versus artemether-lumefantrine for treatment of symptomatic uncomplicated *P. falciparum* infection. All infection was detected by microscopy; Early treatment failure, late treatment failure and adequate clinical and parasitological response was classified based on WHO classification for treatment outcomes.[172]

Table 6.2. Baseline characteristics of the patients

Characteristic	DP arm	AL arm
No. of evaluable patients	144	146
Infecting species at enrolment*		
P. falciparum (%)	122 (84.7)	130 (89.0)
P. falciparum and *P. vivax* (%)	22 (15.3)	16 (10.9)
Median *P. falciparum* density at enrolment per µL (25%-75% range)*	288 (256-520)	288 (256-400)
Female sex (%)	81 (56.3)	79 (54.1)
Age		
Median, years (25%-75% range)	12.04 (8-35)	17.42 (8-36)
< 5 years (%)	20 (13.9)	15 (10.3)
5-14 years (%)	60 (41.7)	56 (38.4)
≥ 15 years (%)	64 (44.4)	75 (51.4)
Temperature ≥ 37.5 °C (%)	15 (10.4)	12 (8.2)
Mean haemoglobin at enrolment, gr/dL (SD)	11.96 (1.9)	11.94 (1.6)
Proportion of anaemic patients (Hb < 10.0 g/dL), %	18 (12.5)	15 (10.3)
History of malaria in previous month (%)	8 (5.6)	13 (8.9)
Use of any prevention (%)	65 (45.1)	54 (36.9)
Use of ITNs (%)	33 (22.9)	29 (19.9)
Use of commercial nets (%)	26 (18.1)	18 (12.3)
Use of repellent (%)	3 (2.1)	4 (2.5)
Use of insecticide spray (%)	2 (1.4)	4 (2.5)
Use of coil repellent (%)	8 (5.6)	4 (2.5)

*Identification and measurement by microscopy.
Abbreviations: ITNs: insecticide-treated bed nets; SD: standard deviation.

6.3.1 Parasite and fever clearance

By 24 hours, 97.9% (141 of 144, 95% CI 94.1 - 99.3) and 98.6% (143 of 146, 95% CI 95.1 - 99.6) of patients in DP and AL groups were afebrile. By 72 hours, 100% (143 of 143) and 98.6% (141 of 143, 95% CI 95.1 to 99.6) in both DP and AL groups were afebrile (Figure 6.2.). Figure 6.3. shows median parasite density at enrolment and during follow-up days 1, 2, and 3 between DP an AL, excluding parasitaemia > 10,000 parasite/µL at enrolment. Overall, 98.6% (142 of 144, 95% CI 95.1 – 99.6) and 98.6% (144 of 146, 95% CI 95.1 – 99.6) of patients in DP and AL cleared parasitaemia within 72 after receiving treatment drugs (*P*=0.82, Figure 6.4.). Mean parasite reduction ratio to 50% (PRR_{50}) and to 90% (PRR_{90}) were 1.05 (SD 0.51) and 1.29 (SD 1.03) days, respectively. There was no difference in parasite clearance times (1.52 days for DP versus 1.45 days for AL, *P*>0.05) between the two groups (Table 6.3.). There was no risk factor associated significantly with presence of parasitaemia on day 3.

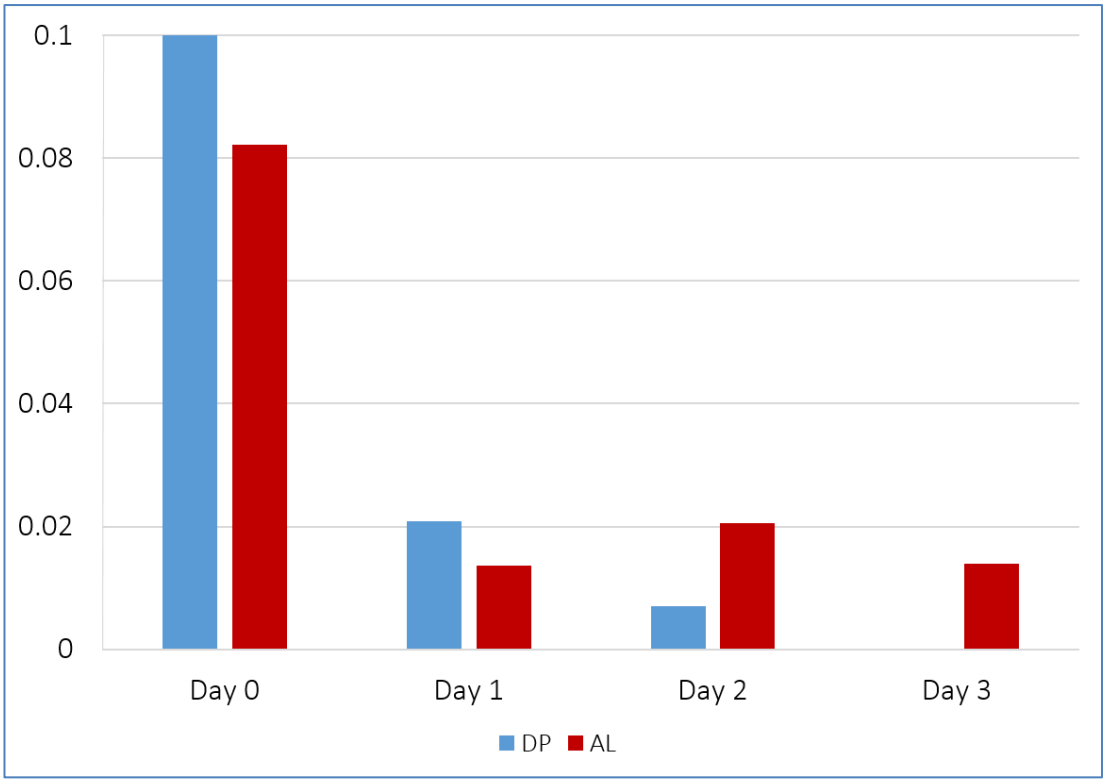


Figure 6.2. Proportion of patients with axillary temperature ≥ 37.5 °C (*n*=144 for DP and *n*=146 for AL).

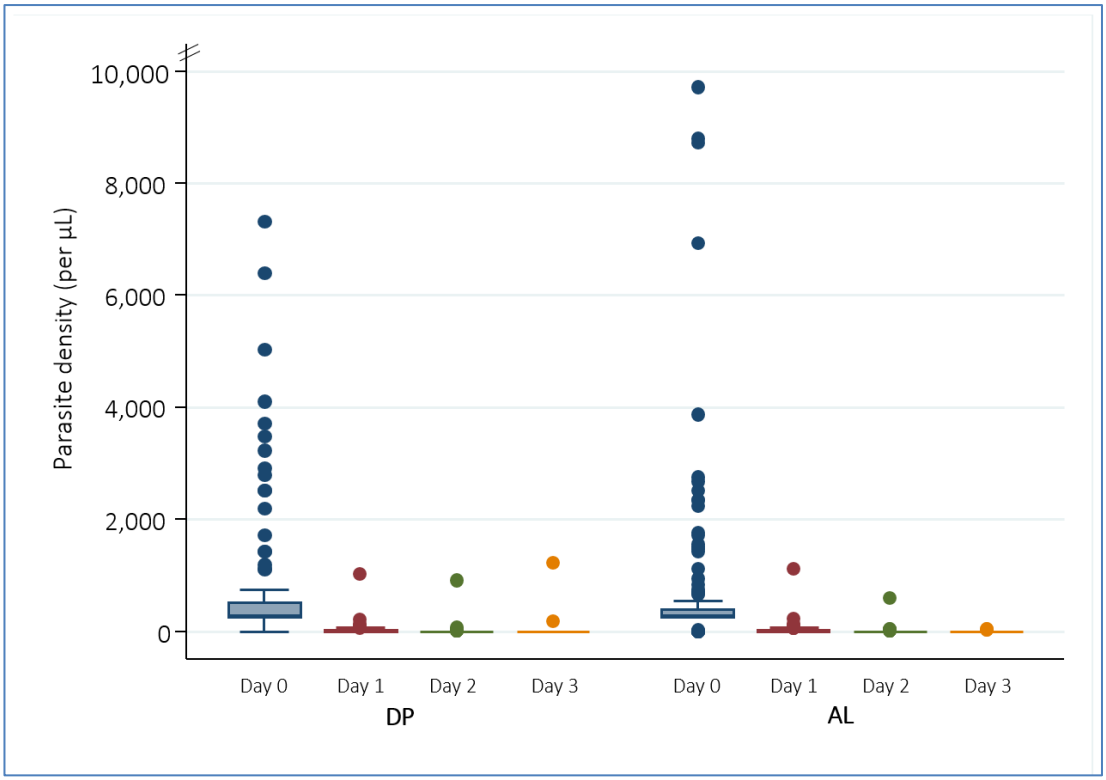


Figure 6.3. Median parasite density for patients with initial parasite density below 10,000 p/µL between two treatment (*n*=139 for DP and *n*=145 for AL) arms by microscopy. Parasite density above 10,000 p/µL are excluded from the analysis (five cases in DP arm and one case in AL arm, all cleared parasitaemia within 24 hours).

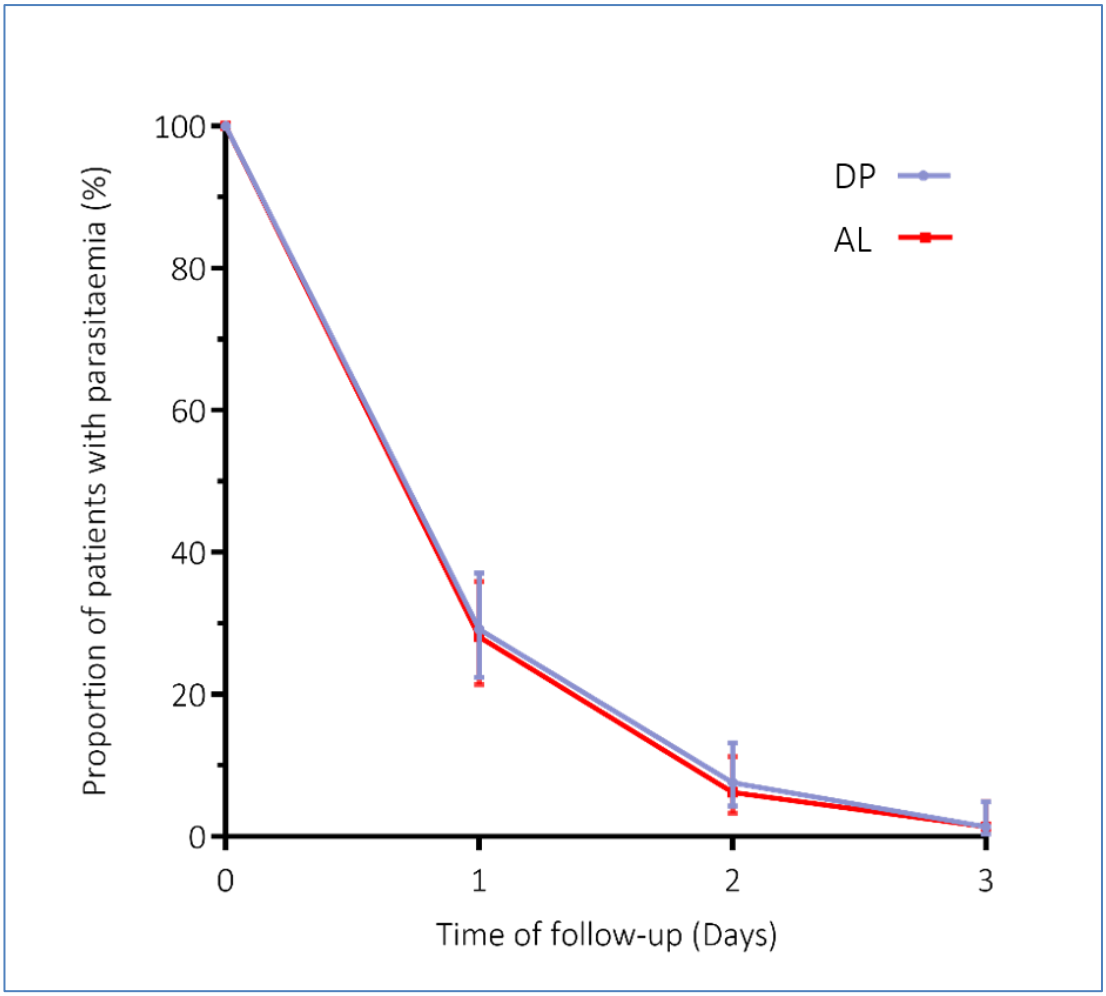


Figure 6.4. Proportion of patients with parasitaemia on days 0, 1, 2 and 3 in the DP (*n*=144) and AL (*n*=146) groups (*P*=0.82).

Table 6.3. Time to parasite clearance measured by microscopy

	DP	AL	*P*
Mean parasite clearance time, days (SD)	1.52 (1.32)	1.45 (0.89)	> 0.05
Positive for sexual parasitaemia on day 3, *n* (%, 95% CI)	2/144 (1.4, 0.4-4.9)	2/146 (1.4, 0.4-4.9)	> 0.05
Mean time to 50% parasite clearance, days (SD)	1.08 (0.72)	1.01 (0.85)	> 0.05
Mean time to 90% parasite clearance, days (SD)	1.32 (1.28)	1.27 (0.71)	> 0.05

6.3.2 Clinical and haematological findings

Both DP and AL were well tolerated and all symptoms improved rapidly following treatment (Figure 6.5.). By 72 hours, 96.5% (139 of 144, 95% CI 92.1 – 98.5) and 97.3% (142 of 146, 95% CI 93.2 – 98.9) patients in DP an AL reported no symptom (Figure 6.6.). Haemoglobin recovery between two groups did not differ significantly at day 28 (*P*=0.87), although when we performed separate analysis for patients who were enrolled with anaemia (Hb < 10.0 gr/dL), we observed significant haemoglobin recovery (*P*<0.001) compared to patients with initial Hb > 10.0 gr/dL (*P*=0.13), (Figure 6.7.). The risk factor associated with anaemia was living in Langkat regency (OR 2.76, 95% CI 1.02 – 6.87).

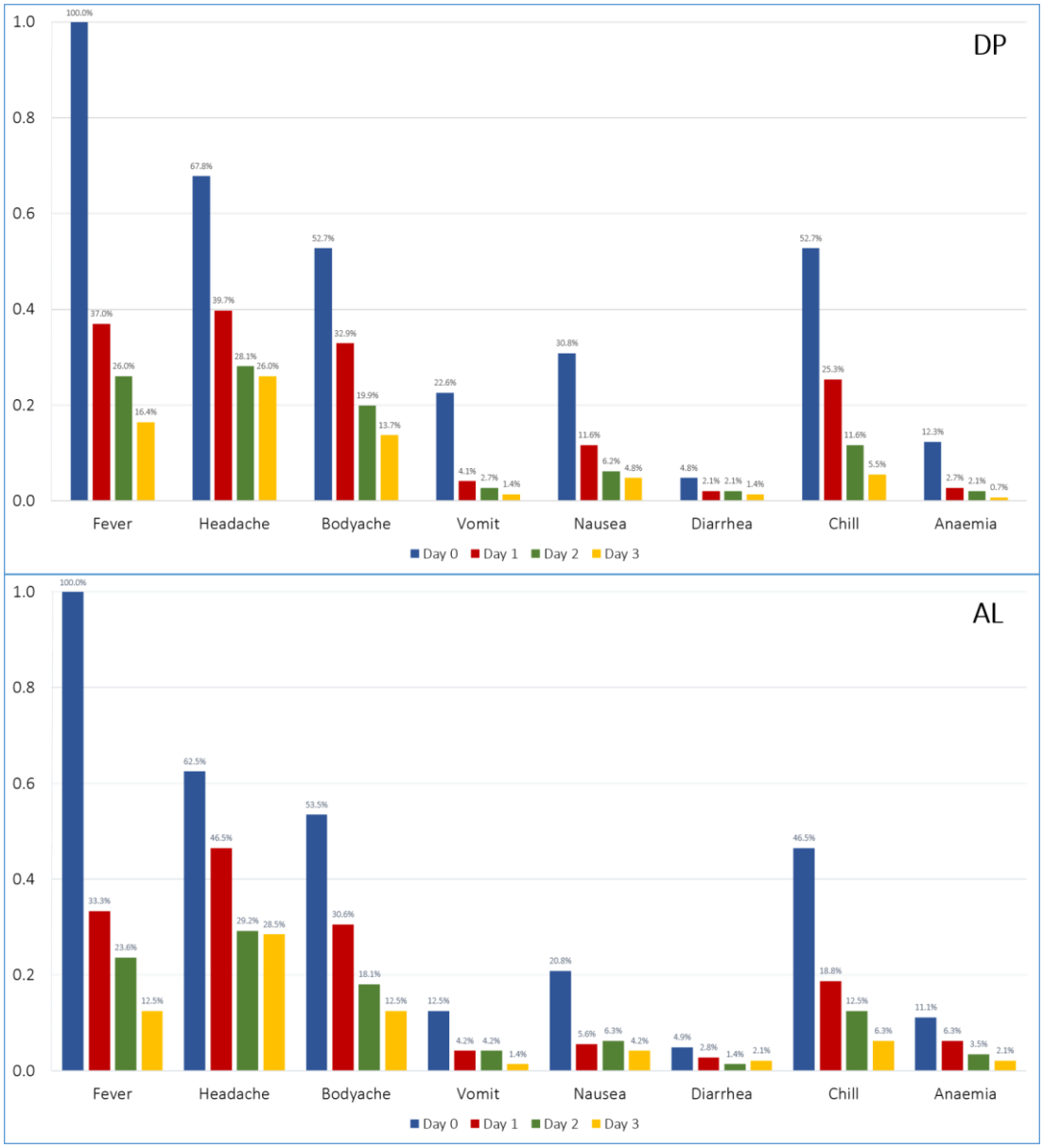


Figure 6.5. Occurrence of symptoms at enrolment and follow-up days in patients receiving DP (*n*=144) and AL (*n*=146). Fever was defined as self-reported fever in the preceding 48 hours on admission or 24 hours during follow-up.

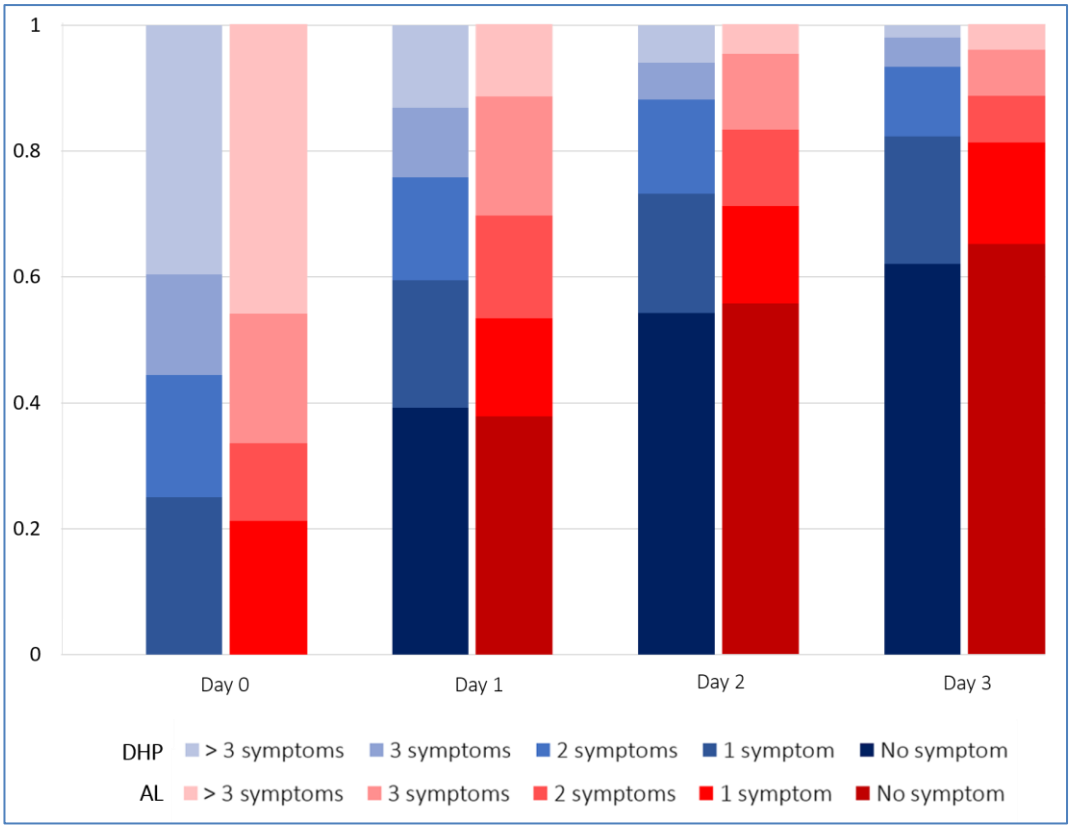


Figure 6.6. Proportion of patients with any symptoms during the first 3 days following treatment.

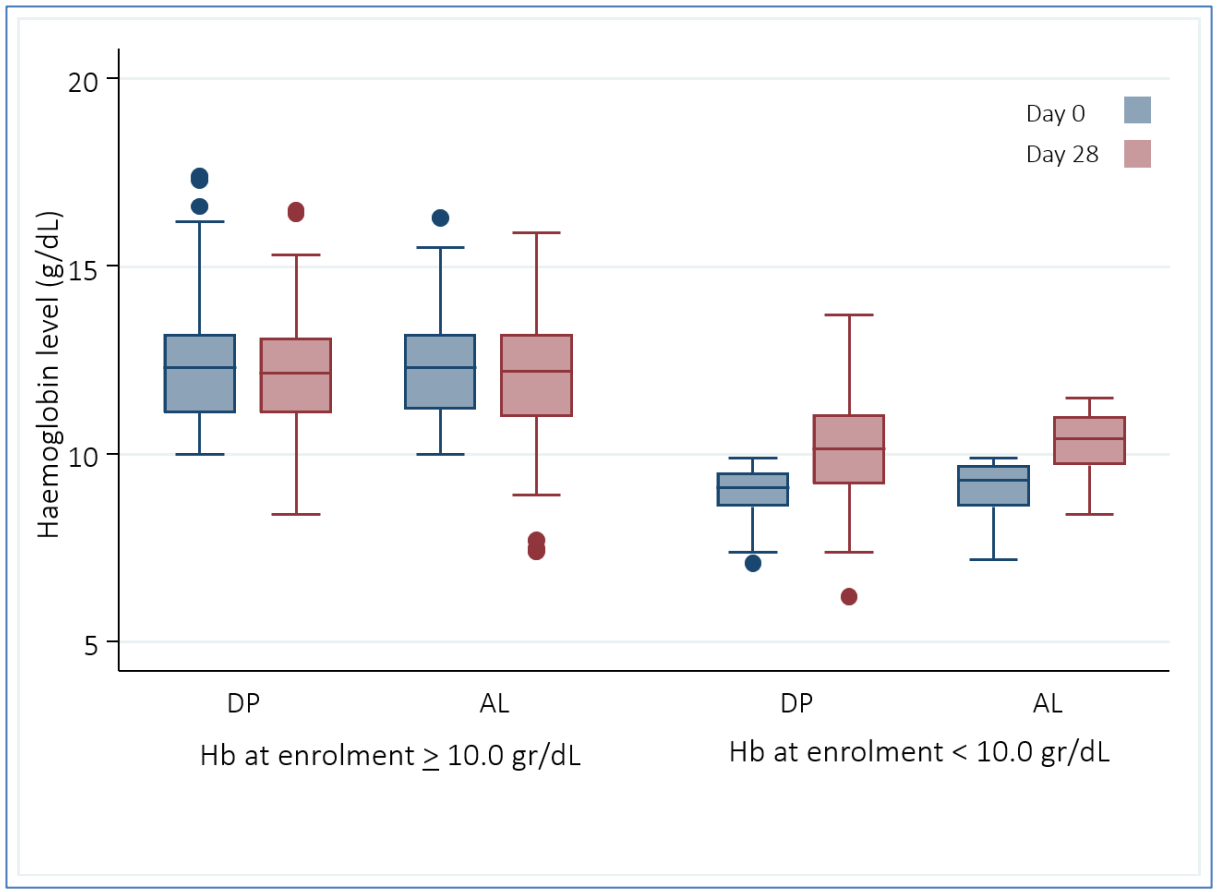


Figure 6.7. Comparisons of haemoglobin levels at enrolment and at day 28 between individuals with Hb ≥ 10.0 gr/dL at enrolment, and individuals with Hb < 10.0 gr/dL at enrolment.

6.3.3 Treatment efficacy

Recurrent infection with either *P. falciparum* or *P. vivax* was detected by microscopy in 37 patients (12.8%) during follow-up, each 23 (15.9%) and 14 (9.6%) in DP and AL groups, respectively. Of those, 1.4% were early treatment failure with 3 cases in DP arm and 1 case in AL arm. One case from each treatment group withdrew on day 3, and two patients treated with DP had detectable day 3 parasitaemia greater than 25% of baseline parasite density. Three among 29 patients with late failure were associated with clinical symptoms, all were in DP treatment group. Two cases of *P. vivax* parasitaemia emerging after treatment occurred in patients with initial diagnosis of *P. falciparum* only, each at day 7 and day 35. One patient with mixed *P. falciparum* and *P. vivax* at enrolment had reappearance of *P. vivax* only at day 42. Mean time to any parasite reappearance was similar in both groups with 15.8 (SD 3.5) days for those receiving DP and 17.4 (SD 4.0) days in AL (*P*=0.29). There was no difference for risk of treatment failures among patients with mono-inflection of *P. falciparum* or co-infection of *P. falciparum* and *P. vivax* at enrolment (13.9% vs 5.3%, *P*=0.14).

The uncorrected efficacy of both treatment at day 42 was 84% (95%CI 76.9 – 89.1) for DP and 90.4% (95% CI 84.4 – 94.2) for AL (Hazard ratio 0.58, 95% CI 0.29 – 1.1, log-rank=0.09) (Figure 6.8.). After correction with PCR, only 1 patient had true recrudescence (Table 6.4.), giving 99.3% (95% CI 95.2 – 99.9) efficacy for DP and 100% for AL (log-rank=0.31) (Figure 6.8.). Using Cox-regression model, predictors for recurrent infection was age between 5 and 15 years old (adjusted Hazard Ratio 1.9, 95% CI 1.35 – 2.75, *P*=0.001), living in South Nias (adjusted Hazard Ratio 3.0, 95% CI 1.39 – 6.51, *P*=0.005) and presence of parasite on day 3 (adjusted Hazard Ratio 21.2, 95% CI 4.38 – 102.58, *P*=0.001) (Table 6.6.).

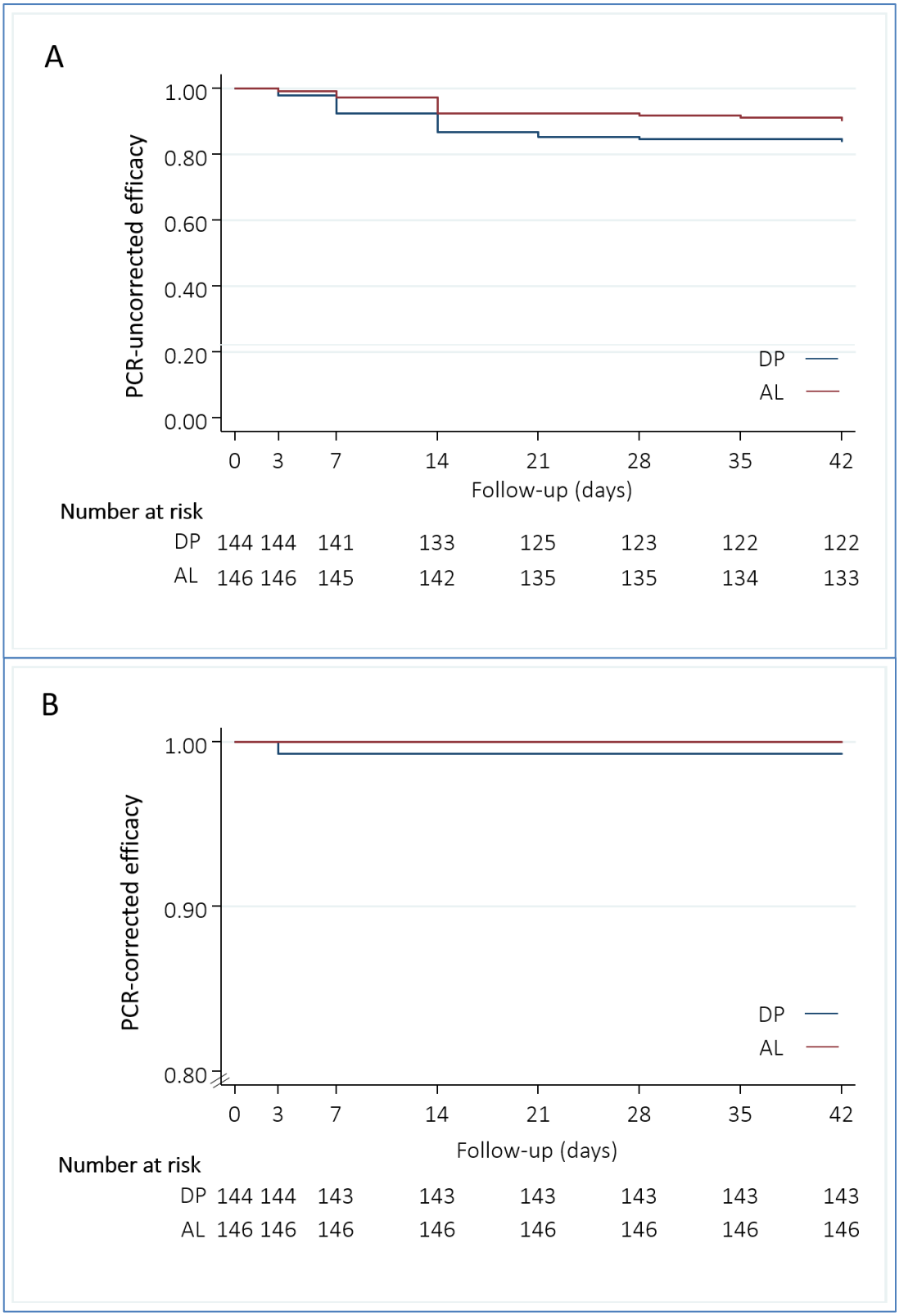


Figure 6.8. (A) Survival rate from recurrent *Plasmodium* infection between DP an AL at day 42 (log-rank=0.09); (B) Survival rate from recrudescence infection between DP and AL at day 42 (log-rank=0.31).

Table 6.4. PCR-uncorrected and PCR-corrected efficacy of DP and AL at day 42

	DP (*n*=144)	AL (*n*-=146)	Hazard Ratio	Log-rank
Uncorrected-PCR				
Early treatment failure				
P. falciparum only	2.1% (3/144, 95% CI 0.7 – 6.3)	0.7% (1/146, 95% CI 0.1 – 4.8)	3.0 (95% CI 0.32 – 29.2), *P*>0.05	0.31
P. vivax only	0%	0%		
Late failure				
P. falciparum only	14.6% (21/144, 95% CI 9.8 – 21.6)	8.9% (13/146, 95% CI 5.3 – 14.9)	1.7 (95% CI 0.85 – 3.4), *P*>0.05	0.12
P. vivax only	1.5% (2/144, 95% CI 0.4 – 5.9)	0.8% (1/146, 95% CI 0.1 – 5.2)	2.14 (95% CI 0.19 – 23.6), *P*>0.05	0.52
Adequate clinical and parasitological outcomes	84% (121/144, 95% CI 76.9 – 89.1)	90.4% (132/146, 95% CI 84.4 – 94.2)	0.58 (95% CI 0.29 – 1.1), *P*>0.05	0.09
Corrected-PCR				
Adequate clinical and parasitological outcomes	99.3% (143/144, 95% CI 95.2 – 99.9)	100% (146/146)		0.31

Table 6.5. Unadjusted Hazard Ratio for *Plasmodium* carriage during post-treatment follow-up

Risk Factor	Hazard Ratio	95% Confidence Interval	*P* Value
Treatment (DP)	1.7	0.89 – 3.37	0.11
Location (Batubara)	4.9	1.39 – 17.35	**0.01**
Location in South Nias	2.4	1.24 – 4.66	**0.009**
Age			
<5 years	0.2	0.03 – 1.42	0.11
5 - <15 years	1.6	1.15 – 2.23	**0.006**
≥ 15 years	0.8	0.66 – 1.04	0.10
Sex (Male)	1.2	0.61 – 2.22	0.64
Temperature > 37.5 at enrolment	3.9	0.53 – 28.22	0.18
History of malaria in previous 1 month	0.8	0.33 – 1.71	0.50
Log parasite density	0.8	0.53 – 1.18	0.21
Monoinfection with *P. falciparum*	0.4	0.09 – 1.51	0.17
Presence of parasites on day 3	7.6	1.83 – 31.86	**0.005**

Table 6.6. Adjusted Hazard Ratio for *Plasmodium* reinfection by day 42

Risk Factor	Hazard Ratio	95% Confidence Interval	*P* Value
Treatment (DP)	1.98	0.98 – 3.98	0.06
Age between 5 and <15 years	1.92	1.35 – 2.75	**0.001**
Location in South Nias	3.00	1.39 – 6.51	**0.005**
Log parasite density	1.36	0.83 – 2.23	0.22
Presence of parasites on day 3	21.2	4.38 – 102.58	**0.001**

One patient with true recrudescence was an 11 year-old girl from South Nias regency diagnosed with *P. falciparum* infection with initial asexual density of 1,120 p/µL. The patient received 3 doses of DP according to her weight (two tablets daily). Her physical examination and nutritional status were normal and her clinical symptom was only fever. On follow-up day 1, asexual parasite density slightly decreased to 1,040 p/µL with emergence of 256 p/µL of gametocytes. On follow-up day 2, asexual and sexual parasites densities became 920 p/µL and 1,240 p/µL, respectively. By day 3, the parasite density was 1,240 p/µL for asexual and 260 p/µL for sexual parasites. Despite slow parasite clearance, there was no clinical complaints recorded on any of the follow-up days. However, due to a delay in the microscopic slide reading, the next data obtained was on follow-up day 7 when patient was subsequently treated with rescue medication (quinine and doxycycline). At this follow-up, parasite density was 480 p/µL for asexual and 1,160 p/µL for sexual parasites before the administration of the rescue treatment. PCR genotyping on day 0, day 3 and day 7 was performed to distinguish recrusdescence from reinfection. DNA amplification targeting *pfmdr1* and *pfk13* genes on day 3 was successful (further discussed in Chapter 7), although amplification was failed by *msp1*, *msp2* and *glurp* genotyping. However, day 7 amplification showed similar band size to the DNA amplified from day 0 by *msp1*, *msp2* and *glurp* genotyping therefore confirming the case as true recrudescence (Figure 6.9.). The parasites negativity results on day 3 by this method of genotyping might be due to the different variants of subpopulations parasites present in the blood or parasites were not present in the peripheral blood during the single sampling reflecting a synchronicity in the subpopulations growth.[367-369] The use of capillary electrophoresis could give an advantage and show more detailed parasite population including minority clones in asymptomatic and symptomatic patients.[370, 371]

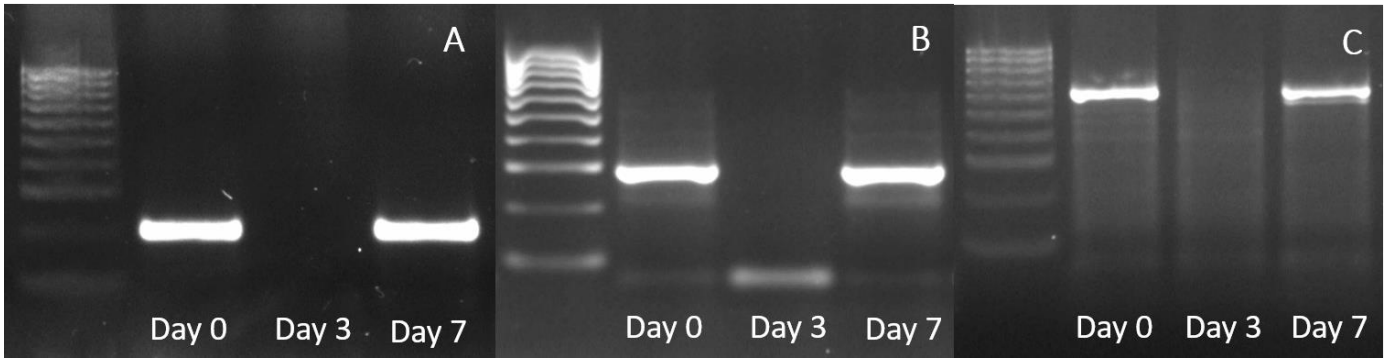


Figure 6.9. One case of early treatment failure confirmed as true recrudescence by *msp1* K1 family genotyping (A), *msp2* FC27 family genotyping (B), and *glurp* genotyping (C).

6.4 Discussion

Our study demonstrated excellent efficacy of both DP and AL for falciparum malaria in western Indonesia. The risk of *P. falciparum* recrudescence at day 42 was very low at 0.7% for DP, while none for AL group. These results are paralleled with previous *in vivo* studies of DP and AL showing efficacy above 90% in both western and eastern parts of the country.[176, 178, 193, 194, 209-212, 217, 218] Artemisinin component kills substantial proportion of parasites rapidly in the first few days, and the partner drug acts to clear the residual parasites and protects individual for longer period. DP was adopted in Indonesia in 2007, initially as the recommended treatment for falciparum malaria in the eastern region but was expanded to the whole country in 2012. AL is available in the private sector.

Despite increasing reports of resistance to artemisinin in the Greater Mekong subregion, as manifested by slow clearing parasites (or parasite half-life longer than 5 hours) and increased day 3 positivity[255], both treatments in our study showed rapid clinical and parasitological cure after treatment. However, our study showed lesser protection given by DP compared to AL as post-treatment prophylaxis. In this study, patients treated with DP had higher risk for recurrent parasitaemia two weeks after receiving their first dose. *P. falciparum* or *P. vivax* recurrence as detected by microscopy were 16% and 9.6% in DP and AL groups, respectively. Weight less than 25 kg has been associated with higher risks of treatment failure in children when using the lower DP dosage recommendation (as in this study), with revised dose of at least 2.5 mg/kg of dihydroartemisinin and 20 mg/kg of piperaquine per day is now being recommended.[372] In this study, 13 out of 37 patients (32.4%) with treatment failures had body weight less than 25 kg. Inadequate blood level of piperaquine (piperaquine blood level < 30 ng/ml at day 7) has also been associated with higher recurrences[211], although some other studies have demonstrated recrudescent infections in patients with adequate piperaquine level.[365] We did not measure piperaquine plasma level in patients in this study.

In western Cambodia where parasite sensitivity to artemisinin is low, emergence of resistance to the partner drugs including piperaquine has been documented which resulted in declining efficacy of ACTs.[118, 365] However, there is no indication that this threat has emerged in west Indonesia or elsewhere in the rest of the country. Treatment failures with ACT have only been reported using the previous first-line antimalarial ASAQ and were not associated with artemisinin resistance.[171, 176] Resistant-parasite genotypes to chloroquine are widely present in this region (as previously described in Chapter 5) due to intensive historical use of

chloroquine, and is suggested to contribute in resistance to amodiaquine. However, this association was not directly studied therefore we cannot confirm the correlation of chloroquine-resistant alleles with amodiaquine failures. Whether the Sumateran parasite genetic background is associated with lesser protection by piperaquine also requires further investigation. Chapter 7 discusses the genetic profile of parasites at baseline, parasites clearance times measured by quantitative PCR and the association of these factors to treatment outcomes.

Malaria transmission in North Sumatera is considered to be unstable and low, with elimination status aimed by 2020.[88] However, our parasite prevalence survey (Chapter 4) prior to the start of the study found PCR positivity rates at 25.2%, 33.5% and 34.8% for Batubara, Langkat and South Nias regencies, respectively.[29] Higher parasite rates in Langkat and South Nias suggest higher transmission risk in these regions. Villages in Langkat and South Nias are remotely located with limited access to drugs. In the present study, all (97.3%) but one patient experiencing recurrent infections were originated from South Nias regency. These data demonstrated that increased exposure in South Nias might have contributed to malaria recurrences. Other predictor for recurrences was children aged between 5 and 15 years, but not in children under 5 years old. This risk is similar to those found in high transmission settings, where younger ages have less developed host immunity to malaria infection.

One patient with true recrudescence after treatment with DP failed to clear parasites until rescue medication was given at day 7. All study drugs were administered according to weight-based recommendation and given concomitantly with food as drug absorption is increased by fatty food.[373] Drug administrations were also directly observed by study staff. The patient's nutritional status was within normal range, and there was no other complaint that could be related to poor absorption. Nevertheless, parasite density within the first 72 hours was only slightly reduced suggesting poor drug absorption or low sensitivity to artemisinin to cause treatment failure. However, the parasites were still not eliminated by day 7 indicating partner medicine failed also to clear parasites. There is a strong suggestion that inadequate drug level plays a role in the treatment failure. However, as we did not measure piperaquine blood level in patients, it is difficult to confirm the cause of treatment failure in this case. Assessment on genotypic profile of the parasites including analysis of *pf*k13-propeller domain and copy number of *pflasmepsin2* as the resistance markers for this drug combination will provide explanation on the contribution of genetic factors on drug efficacy. Chapter 7 discusses further the association of genetic profiles and treatment outcomes.

Other limitation of the study is that we included patients with mixed infection, hence not allowing true assessment of *P. falciparum* infection alone. How further multiple infections contribute in treatment outcomes still has to be studied. However, epidemiological studies have shown that multiple infections of *P. falciparum* with *P. vivax* or *P. malariae* are often occurred together.[29, 193] In Indonesia, DP is the drug of choice for all malaria species and also given in mixed infections.[128] Therefore, inclusion of mixed infections reflects routine clinical practice. In this study, only small proportion (13.1%) of mixed infections were enrolled. We found no association between species multiplicity at enrolment and treatment outcomes. True recrudescence occurred in a patient diagnosed with single *P. falciparum* infection, and only 2 out of 37 patients with recurrent infections had mixed infections with *P. vivax*. We did not perform specific analysis for mixed *P. falciparum* and *P. vivax* infections due to small numbers of cases.

In conclusion, our study provides additional information on the extent of artemisinin resistance in Southeast Asia beyond Greater Mekong subregion. We show clear evidence on high efficacy of two ACTs 5 years after their deployment in west Indonesia. In history, chloroquine-resistant parasites took about 10 years before spreading eastward from its origin in Cambodia to Indonesia.[141] However, whether artemisinin resistance will spread following the same historical path or emerge independently in this region remains unknown. Routine monitoring by performing regular drug efficacy study supported by molecular markers analysis are the key strategy to halt the development of resistance to ACTs in this corner of Southeast Asia.

Chapter 7

RESULTS 4

7 RESULTS

Molecular detection and characterisation of recurrent parasitaemia following ACT treatment

7.1 Introduction

Resistance to antimalarial drugs has been a constant challenge for malaria control and elimination programmes. Currently artemisinin-based combination therapies (ACTs) are the first-line treatment in almost all malaria endemic countries.[1, 139] However, reduced susceptibility of *Plasmodium falciparum* toward this combination (artemisinin and the partner drugs) has been reported in western Cambodia and other countries in the Greater Mekong subregion (GMS).[118, 255] The drug failures appearance is so far contained to this region[302], but stresses the importance of regular monitoring on drug efficacy in all endemic regions, and early identification of genetic changes in the parasites.

P. falciparum polymorphisms associated with reduced sensitivity to ACTs have been characterised by mutations in the transporter genes *pfcrt* and *pfmdr1*, and the amplification of *pfmdr1* gene copy number and *pfplasmepsin2* gene copy number.[199, 268, 269, 294, 295, 359, 374] Certain mutations in the *pfk13* have been identified as markers for artemisinin resistance, the currently most potent antimalarial. Polymorphisms in the kelch propeller domain of this gene are associated with delayed parasite clearance determined by microscopy[268], and residual parasites surviving artemisinin pose a risk to facilitate selection of parasites to the partner drugs. Moreover, subpatent residual parasites detected on day 3 by quantitative polymerase chain reaction (qPCR) have also been suggested to contribute to a higher risk of recurrences at day 28 and day 42, which was potentially contributing to parasite transmission to mosquitoes.[188, 196]

Previous studies from North Sumatera revealed that the majority of *P. falciparum* isolates from this region harbour the *pfcrt* mutant 76T and *pfmdr1* mutant 86Y.[107, 109]

Nevertheless, despite the abandonment of chloroquine and the utilisation of ACT, artesunate-amodiaquine (ASAQ), since 2004 in this region, our study still documented similar genetic signatures of *P. falciparum* population as previously reported (see Chapter 5). Most *P. falciparum* parasites in our three study sites were shown to harbor the *pfcrt* SVMNT and *pfmdr1* 86Y and 184Y, and a small proportion of isolates also carried mutant *pfk13* (see Chapter 5). This is the first study to report the presence of this kelch13 mutant parasites in western Indonesia. However, the impact of this mutation on ACT in the background of SVMNT and *pfmdr1* YY parasites needs further investigation.

In this study, we evaluated the responses of *P. falciparum* parasites to dihydroartemisinin-piperaquine (DP) and artemether-lumefantrine (AL) treatment (Chapter 6). In this chapter, we characterise the genetic profiles of parasites prior to treatment, we assess parasite clearance dynamics following ACTs treatment, and determine the association between baseline polymorphisms, parasite clearance rate and parasite genotypes from recurrent isolates on days 28 and 42 to evaluate post-treatment selection *in vivo*. Comparisons are made between the two treatment arms and among study sites.

7.2 Methods

7.2.1 Samples collection

Samples were obtained from patients enrolled in the *in vivo* drug efficacy study collected between January and June 2015 in North Sumatera province, Indonesia. Inclusion and exclusion criteria for enrollment were described in Chapter 6. Patients with *P. falciparum* malaria were randomised to receive either DP or AL, and patients were followed-up for 42 days after treatment. Samples were then analysed for drug resistance markers related to the study drugs including *pfcrt*, *pfmdr1*, *pfk13*, and *pfmdr*1 copy numbers. Parasite clearance times following treatment were also measured by qPCR assay. End-point outcome was the cumulative risk of *P. falciparum* recurrences at day 42 as detected by polymerase chain reaction (PCR).

The study protocol was approved by the ethics committee of the London School of Hygiene and Tropical Medicine, United Kingdom (London, United Kingdom; 8504-01) and by the ethics committee of University of Sumatera Utara, Indonesia (Medan, Indonesia; 401/KOMET/FKUSU/2014). The study was registered at ClinicalTrials.gov under number NCT02325180.

7.2.2 Characterisation of parasite polymorphisms

7.2.2.1 Polymorphisms in the *pfcrt*, *pfmdr1* and *pfk*13

Parasite DNA was extracted from blood spots on 3MM Whatman filter papers and from rapid diagnostic tests following either the Chelex or QIAsymphony extraction methods.[320] Protocols for amplification and sequencing of *pfcrt*[270], *pfmdr1*[273] and *pfk13*[268] genotyping were as described in the methods section of chapter 5. Codons 72 to 76 in *pfcrt,* codons 86, 184, 1034, 1042 and 1246 in *pfmdr1*, and propeller domain of *pfk13* are of particular interest. The proportion of each haplotype was calculated and compared between the two treatment arms and study sites.

7.2.2.2 *Pfmdr1* copy number

Pfmdr1 gene copy number was assessed by a multiplex real-time qPCR Rotor-gene® Q thermocycler (Corbett Research, Australia) following a protocol by Price with modification.[199] Each isolate was tested in duplicate with control DNA from *P. falciparum* lines 3D7 and 7G8 (1 copy of *pfmdr1* each) and Dd2 (2 copies of *pfmdr1*) included in each experiment. The primers and probes for the amplification are provided in Table 7.1. Mastermix reaction was prepared in 25 µL containing 5 µL of template DNA, NH_4 buffer, 5.5 mM $MgCl_2$, 300 µM deoxynucleotides (dNTPs), 300 nM for each forward and reverse primer, 100 nM of each probe, 8% glycerol, and 1 unit of BIOTAQ (Bioline, UK). The amplification cycling conditions were 95 °C for 6 minutes as initial denaturation, followed by 45 cycles of 95 °C for 15 seconds and 58 °C for 1 minute.

Table 7.1. Primers and probes for *pfmdr1* copy number amplification[199]

Gene	Primer	5' Fluorophore	Sequence	3' Quencher
Pfmdr1	*Pfmdr1*-1F		5'-TGC ATC TAT AAA ACG ATC AGA CAA A-3'	
	Pfmdr1-1R		5'-TCG TGT GTT CCA TGT GAC TGT-3'	
	Pfmdr1-probe	FAM	5'-TTT AAT AAC CCT GAT CGA AAT GGA ACC TTT G-3'	BHQ2
ß-tubulin	ß-tubulin-1F		5'-TGA TGT GCG CAA GTG ATC C-3'	
	ß-tubulin-1R		5'-TCC TTT GTG GAC ATT CTT CCT C-3'	
	ß-tubulin-probe	JOE	5'-TAG CAC ATG CCG TTA AAT ATC TTC CAT GTC T-3'	BHQ2

The qPCR assay generates fluorescent signals for the target (*pfmdr1*) and reference (*P. falciparum ß tubulin*) genes. The threshold was set above negative controls run in the same assay. The cycle threshold (C_t) is determined when the amplification of target (Ct_G) and reference (Ct_R) genes cross the set threshold. The concentration of genomic DNA was then estimated using the formula $\Delta C_t = Ct_R - Ct_G$. This value was plotted against the log of initial DNA concentration. Then the formula $\Delta\Delta C_t = Ct_E - Ct_B$ was calculated, with Ct_E as the experimental C_t and Ct_B as the baseline C_t. Relative expression was then calculated as $2^{-\Delta\Delta Ct}$ to account for the exponential properties of PCR. Results were rejected if $\Delta\Delta C_t$ spread > 1.5 or C_t values >35. Value above 1.5 classified as multicopy.[199]

7.2.3 Parasite clearance times

Parasite density at day 0, 1, 2 and 3 were also measured with Rotor-gene® Q thermocycler (Corbett, Australia) using the method described by Beshir.[321] Reaction mixture of 25 µL was prepared containing 5 µL of template DNA, 10X NH_4 buffer, 5.5 mM $MgCl_2$ buffer, 300 µM dNTPs, 300 nM forward and reverse primer, 200 nM of each probes (Eurofin), 1.0 unit of BIOTAQ DNA polymerase (Bioline, UK). Cycling conditions were 95 °C for 6 minutes, followed by 40 cycles of 95 °C for 15 seconds and 68 °C for 1 minute. Primers and probes for *Plasmodium* tRNA methionine (PgMET) and human β tubulin gene (HumTuBB) amplification are provided in Table 7.2. All samples were run in duplicate with the WHO International Standard as control for *P. falciparum* DNA.[335] Difference of parasite target gene (PgMET) C_t and the human reference gene (HumTuBB) C_t was calculated with the formula of $\Delta C_t = C_{t\ PgMET} - C_{t\ HumTuuBB}$. The change over time in C_t was calculated using formula $\Delta\Delta C_{t(n)} = \Delta C_{t(n)} - \Delta C_{t(0)}$, with *n* as time after treatment and 0 as time before treatment. Parasite density was then measured against the calibrator sample (day 0) using formula $2^{-\Delta\Delta Ct}$.[321] Using this model, parasite prevalence and relative density over the first 72 hours after treatment were determined. Parasite reduction ratio to 50% (PRR_{50}) and to 90% (PRR_{90}) were also determined.

Table 7.2. Primers and probes for parasite clearance time assay[321]

Primer	5' Fluorophore	Sequence	3' Quencher
PgMET_F1		5'-TGAAAGCAGCGTAGCTCAGA-3'	
PgMET_R2		5'-CGCGTGGTTTCGATCCACG-3'	
PgMET_pB	FAM	5'-GGGGCTCATAACCCCCAGGA-3'	BHQ2
HumTuBB_F2		5'-AAGGAGGTCGATGAGCAGAT-3'	
HumTuBB_R2		5'-GCTGTCTTGACATTGTTGGG-3'	
HumTuBB_Joe	JOE	5'-TTAACGTGCAGAACAAGAACAGCAGCT-3'	BHQ2

7.2.4 Study outcomes

7.2.4.1 Microscopy-detected treatment failures

Samples with detectable parasites by microscopy on follow-up days which met the criteria for treatment failure according to the WHO classification[172] were further analysed. A conventional nested PCR amplification targeting the 18S rRNA and *sicavar* genes were performed for species determination.[29, 332] Genetic characterisations were also performed on samples with PCR-corrected confirmed recrudescence.[199, 268, 270, 273]

7.2.4.2 PCR-detected recurrences

To further evaluate post-prophylaxis protection by both ACTs, we performed molecular screening to detect any subpatent recurrences on samples collected on day 28 and day 42 or on any last day samples available. All available samples were screened using qPCR Rotor-gene® Q assay (Corbett, Australia) targeting parasite PgMET gene following similar protocol in the parasite clearance times assay (see Chapter 7.2.3.). This assay amplifies a fragment of PgMET in all human *Plasmodium* species.[321] Hence, samples with Ct value $\leq$ 35 were further analysed for *Plasmodium* speciation using nested PCR with a target of 18S rRNA gene.[332] Additional nested PCR targeting *pfmdr1* was also performed to increase the sensitivity to detect *P. falciparum* recurrences.

7.2.5 Statistical analysis

Data analysis was done using Stata IC (version 15, StataCorp, TX, USA). Logistic regression analysis was used to estimate the association between baseline genotypes and day 3 positivity by qPCR or the event of recurrences. Odds ratio and 95% confidence intervals were estimated. Treatment outcomes were determined by calculating the cumulative of recurrences with *P. falciparum* at day 42 using Kaplan Meier survival analysis and log-rank test. Cox-regression analysis was used to predict risk factor for recurrences. *P* values of $\leq$ 0.05 are considered statistically significant.

7.3 Results

A total of 302 patients with uncomplicated *P. falciparum* infection were enrolled in the drug efficacy study. Baseline characteristics of patients were described in Chapter 6. *Post hoc* PCR assays targeting the 18S rRNA[332], *pfmdr1*[273] and *sicavar*[29] gene were performed to confirm the presence of *P. falciparum* parasites and to identify other *Plasmodium* species in each isolate prior to treatment (see Section 4.2.). Surprisingly, in Batubara, Langkat and South Nias regencies, parasite DNA was detected in 91.7% (33 of 36), 72.1% (31 of 43), and 58.8% (131 of 223) of samples (Table 7.3.), respectively. Of these, only 117 (38.8%) samples were positive for *P. falciparum* infection, 57 (48.8%) in the DP-treated arm and 60 (51.2%) in the AL-treated arm.

Table 7.3. Confirmation of *Plasmodium* species by PCR among enrolled patients

PCR diagnosis*	Batubara, *n* (%)	Langkat, *n* (%)	South Nias, *n* (%)	Overall, *n* (%)
Negative	3/36 (8.3)	12/43 (27.9)	92/223 (41.3)	107/302 (35.4)
P. falciparum	20/36 (55.6)	23/43 (53.5)	34/223 (15.2)	77/302 (25.5)
P. falciparum + other *Plasmodium* species	10/36 (27.8)	2/43 (4.7)	28/223 (12.6)	40/302 (13.2)
Other *Plasmodium* species	3/36 (8.3)	6/43 (13.9)	69/223 (30.9)	78/302 (25.8)

*Diagnosis was determined by PCR assays targeting the 18S rRNA, *Pfmdr1*, *sicavar* gene (see text).

7.3.1 Genetic markers of parasites at baseline

Genotyping of the *pfcrt* gene using qPCR assay was successful in 60.1% (71 of 117; 34 isolates in DP, 37 isolates in AL) of samples harbouring *P. falciparum* infection. Mutant haplotype encoding SVMNT was the most prevalent haplotype in both DP (22 of 34, 64.7%) and AL groups (30 of 37, 81.1%). Wild-type CVMNK was present in 32.4% (11 of 34) and 16.2% (6 of 37) isolates in DP and AL treatment arms, while CVIET was less common with prevalence of 8.8% (3 of 34) and 8.1% (3 of 37) in DP and AL groups, respectively. 20.6% of DP-treated samples and 16.2% of AL-treated samples harboured mixed wild-type and mutant haplotypes. Stratified by regency, the *pfcrt*-SVMNT remained the most observed haplotype in each site (Table 7.4., Figure 7.1.).

Table 7.4. Baseline prevalence of *pfcrt* polymorphisms in DP and AL treatment arms according to site

Pfcrt 72-76 haplotype	Batubara, *n* (%)		Langkat, *n* (%)		South Nias, *n* (%)		Overall, *n* (%)
	DP	AL	DP	AL	DP	AL	
CVMNK	0/25 (0)	0/25 (0)	1/22 (4.5)	0/22 (0)	3/24 (12.5)	0/24 (0)	4/71 (5.6)
CVIET	0/25 (0)	0/25 (0)	0/22 (0)	0/22 (0)	2/24 (8.3)	2/24 (8.3)	4/71 (5.6)
SVMNT	11/25 (44.0)	12/25 (48.0)	6/22 (27.3)	9/22 (40.9)	4/24 (16.7)	8/24 (33.3)	50/71 (70.4)
CVMNK-SVMNT	0/25 (0)	1/25 (4.0)	4/22 (18.2)	1/22 (4.5)	2/24 (8.3)	3/24 (12.5)	11/71 (15.5)
CVMNK-CVIET-SVMNT	1/25 (4.0)	0/25 (0)	0/22 (0)	1/22 (4.5)	0/24 (0)	0/24 (0)	2/71 (2.8)

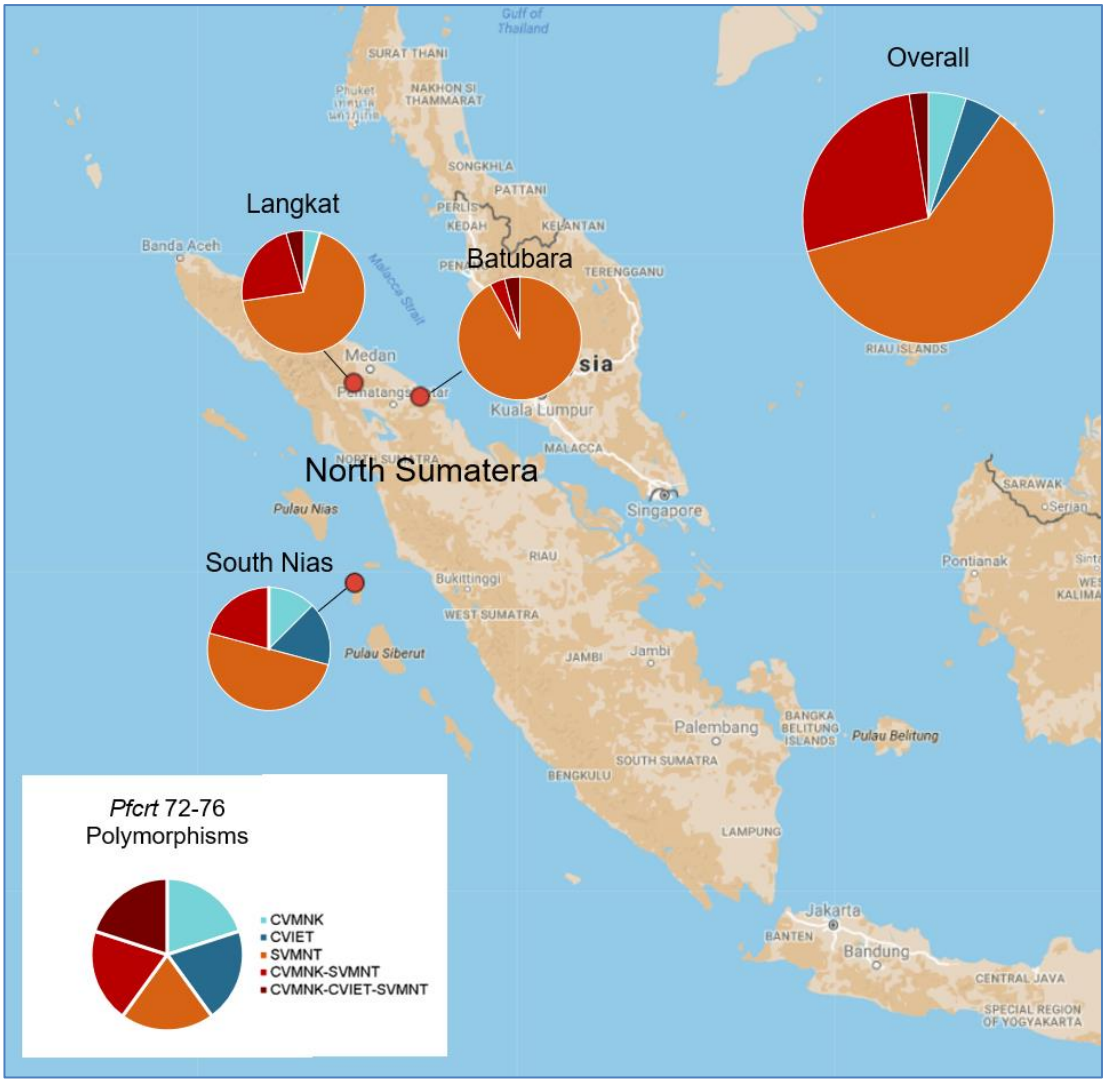


Figure 7.1. Baseline *pfcrt* 72-76 polymorphisms according to study sites (Overall *n*=71, *n*=25 for Batubara, *n*=22 for Langkat, and *n*=24 for South Nias).

Amplification of the *pfmdr1* gene was successful in 82.1% (96 of 117) isolates. Pre-treatment prevalence of *pfmdr1* 86, 184, 1034, 1042 and 1246 alleles are presented in Table 7.5, with mutant 86Y and wild-type 184Y were shown as the most prevalent. The wild-type 86 occurred in 34.0% and 21.3% of samples in DP and AL, respectively, and rare mutant 86S was also present in two isolates in the AL group. The genotypes from alleles 86 and 184 were then collated into haplotype (see Chapter 5). Mixed alleles were included in the analysis, assuming one of the haplotypes of interest occurred. Our analysis showed haplotype YY of *pfmdr1* gene was the most predominant haplotype with proportions of 65.3% (32 of 49) in DP and 76.6% (36 of 47) in AL, respectively. Isolates harbouring haplotype NF and NY were 6.1% and 22.5% in DP group, and 4.3% and 14.9% in AL group. Haplotype-YY was also found to be the most predominant in Batubara and Langkat, while haplotype NY was equally common as mutant haplotype-YY in South Nias. The proportions of these haplotypes according to site are shown in Table 7.6 and Figure 7.2.

Table 7.5. Baseline prevalence of *pfmdr1* alleles in DP and AL treatment arms

Pfmdr1	DP, *n* (%)				AL, *n* (%)			
	Wild-type	Mutant	Other	Mixed	Wild-type	Mutant	Other	Mixed
N86Y	16/47 (34.0)	30/47 (63.8)	0/47 (0)	1/47 (2.1)	8/45 (21.3)	34/45 (75.6)	2*/45 (4.5)	1/45 (2.2)
Y184F	42/46 (91.3)	4/46 (8.7)	0/46 (0)	0/46 (0)	41/45 (91.1)	4/45 (8.9)	0/45 (0)	0/45 (0)
S1034	22/22 (100)	0/22 (0)	0/22 (0)	0/22 (0)	25/25 (100)	0/25 (0)	0/25 (0)	0/25 (0)
N1042	22/22 (100)	0/22 (0)	0/22 (0)	0/22 (0)	25/25 (100)	0/25 (0)	0/25 (0)	0/25 (0)
D1246Y	22/22 (100)	0/22 (0)	0/22 (0)	0/22 (0)	21/21 (100)	0/21 (0)	0/21 (0)	0/21 (0)

*86S was present in two samples in the AL group.

Table 7.6. Baseline prevalence of *pfmdr1* 86/184 haplotypes in DP and AL treatment arms according to site

Pfmdr1 haplotype	Batubara, *n* (%)		Langkat, *n* (%)		South Nias, *n* (%)		Overall, *n* (%)
	DP	AL	DP	AL	DP	AL	
86N/184Y	0/27 (0)	0/27 (0)	0/22 (0)	0/22 (0)	11/43 (25.6)	7/43 (16.3)	18/92 (19.6)
86N/184F	0/27 (0)	0/27 (0)	1/22 (4.5)	0/22 (0)	2/43 (4.7)	2/43 (4.7)	5/92 (5.4)
86Y/184Y	13/27 (48.2)	14/27 (51.8)	10/22 (45.5)	10/22 (45.5)	9/43 (20.9)	12/43 (27.9)	67/92 (72.8)
86S/184F	0/27 (0)	0/27 (0)	0/22 (0)	1/22 (4.5)	0/43 (0)	1/43 (2.3)	2/92 (2.2)

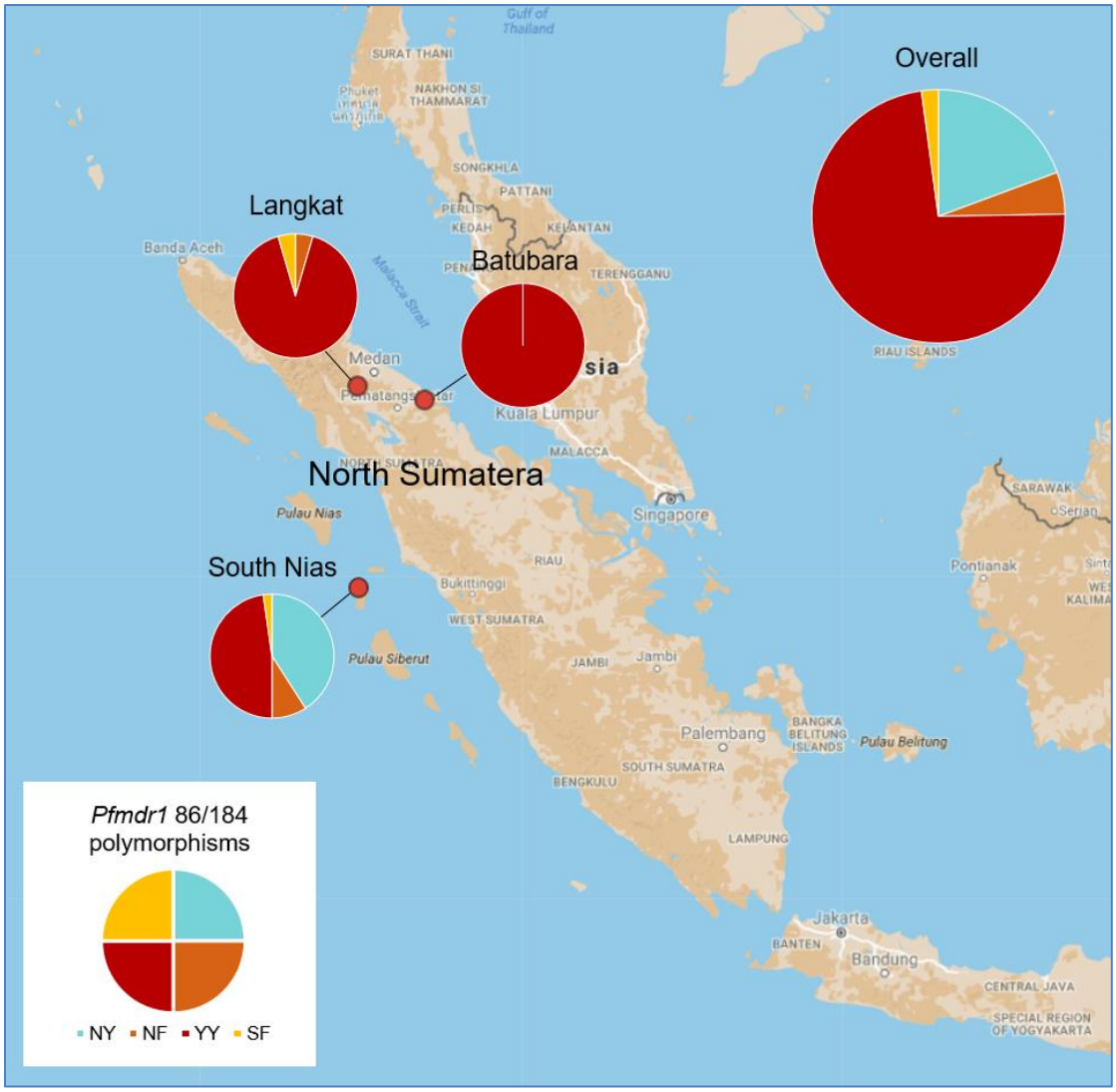


Figure 7.2. Baseline *pfmdr1* haplotypes according to study sites (Overall *n*=92, *n*=27 for Batubara, *n*=22 for Langkat, and *n*=43 for South Nias).

Pfmdr1 copy number was successfully determined in 35 samples and gene amplifications were observed in 14 (40%) samples. There was no difference in the proportion of single copy *pfmdr1* between location or treatment (Table 7.7.).

Table 7.7. Proportion of single and multicopy numbers of *pfmdr1* gene according to treatment arms

Copy numbers	Single copy, *n* (%)			Multi copy, *n* (%)		
	Batubara	Langkat	South Nias	Batubara	Langkat	South Nias
DP, n (%)	9/11 (81.8)	1/11 (9.1)	1/11 (9.1)	1/5 (20.0)	0/5 (0)	4/5 (80.0)
AL, n (%)	3/10 (30)	3/10 (30)	4/10 (40)	6/9 (66.7)	1/9 (11.1)	2/9 (22.2)

Genotyping analysis on *pfk13* propeller domain revealed high proportion of wild-type parasites. All isolates (38 of 38, 100%) in DP arm and 91.9% (34 of 37) in AL arm harboured artemisinin-sensitive parasites. Three isolates (8.1%) in the AL group had a mixed wild-type and mutant K13 carrying T474A mutation (Figure 7.3.), these were detected in Batubara and Langkat (Table 7.8. and Figure 7.4.). All three mutant K13 isolates had a background of *pfcrt*-SVMNT haplotype. Two of the three had *pfmdr1* haplotype YYSND on the interest alleles, and one sample had SFSND haplotype. None of the known K13 mutations associated with artemisinin resistance was detected in any of our samples.

Overall, the majority of the samples harboured the *pfcrt* SVMNT and *pfmdr1* YYSND haplotypes. These haplotypes were also observed as the majority haplotypes in Batubara (96% for *pfcrt* and 100% for *pfmdr1*) and Langkat (72.7% for *pfcrt* and 86.9% for *pfmdr1*). In contrast, only 50% and 44.7% of patients in South Nias displayed these haplotypes at baseline. These findings are similar to our previous observations described in Chapter 5.

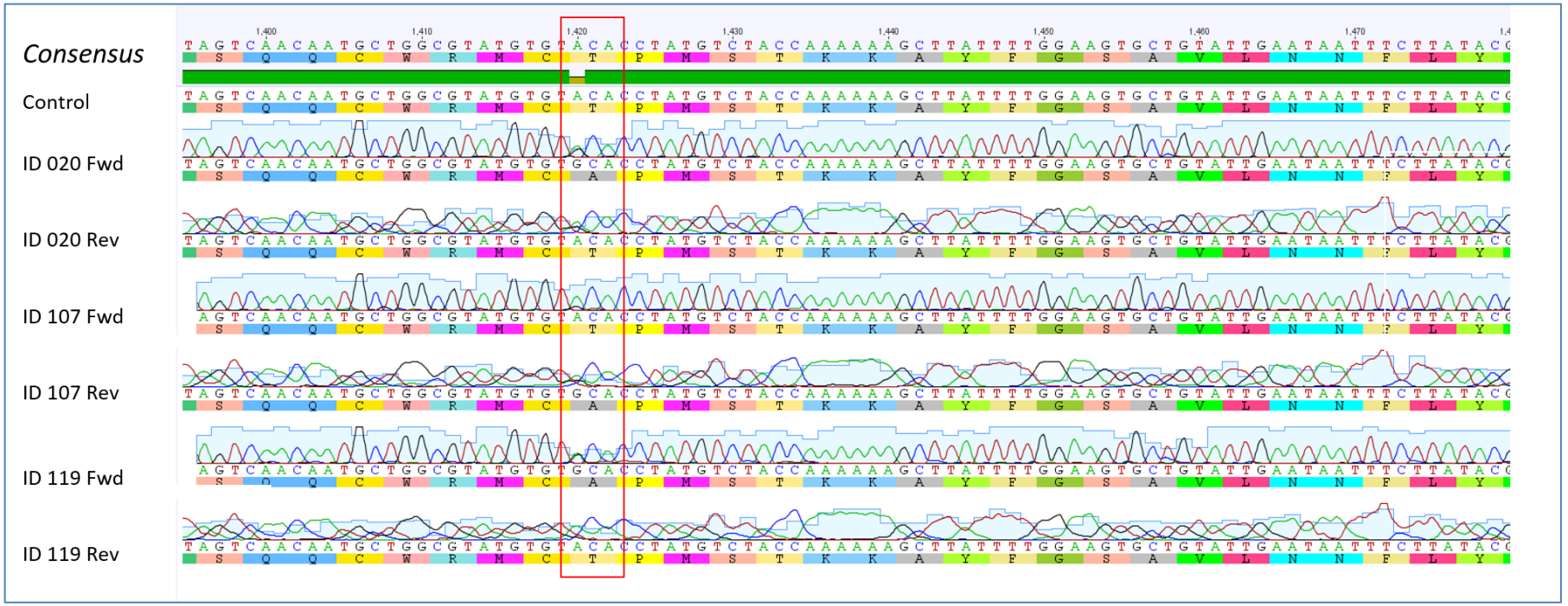


Figure 7.3. Sequences of propeller domain of *pfk13* in three patients with mixed wild-type and mutant at allele T474A.

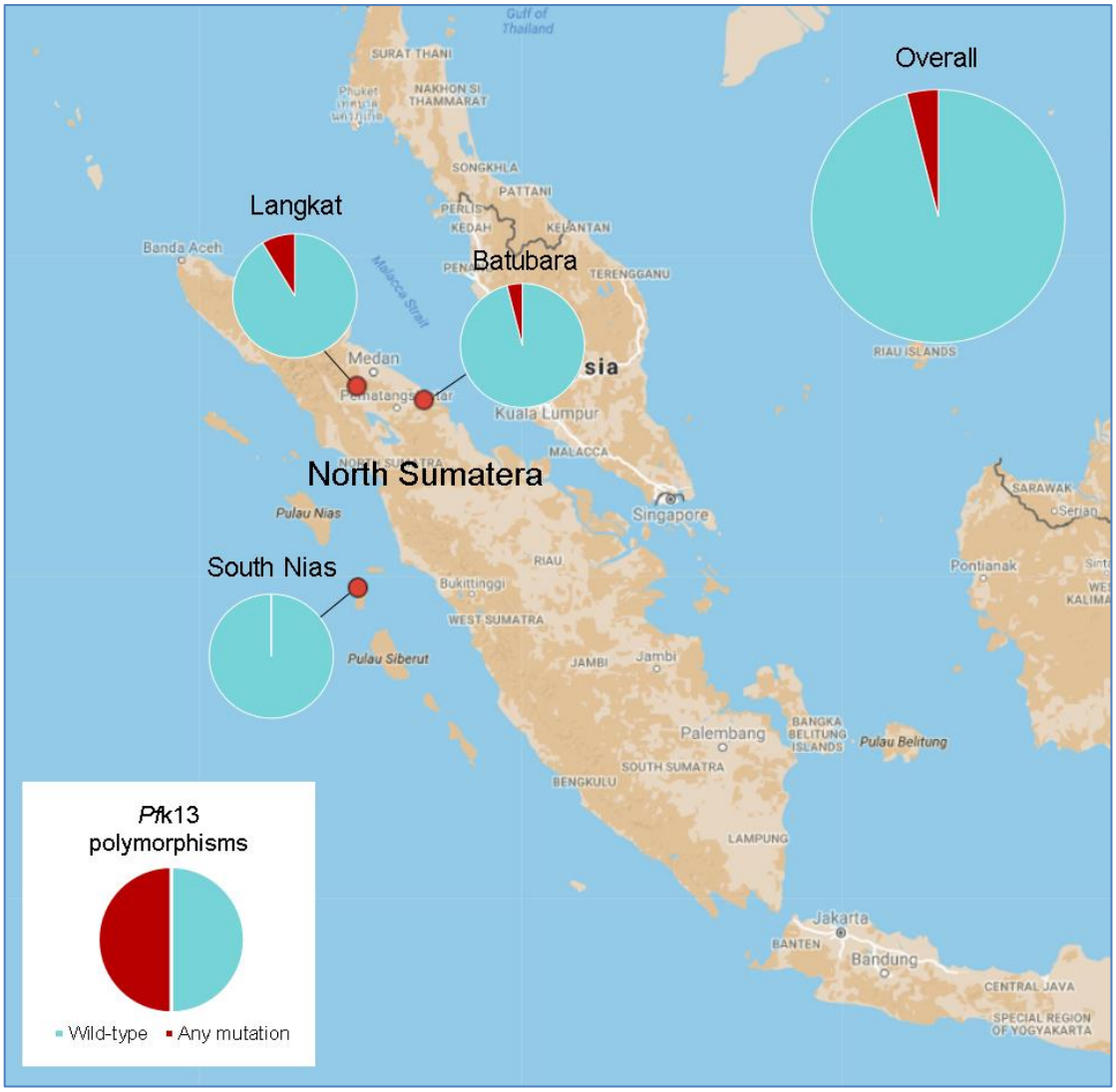


Figure 7.4. Baseline *pfk13* propeller domain polymorphisms according to study sites (Overall *n*=75, *n*=25 for Batubara, *n*=23 for Langkat, and *n*=27 for South Nias).

Table 7.8. Baseline prevalence of *pfk13* wild-type and mutant alleles in DP and AL treatment arms according to site

*Pf*kelch13	Batubara, *n* (%)		Langkat, *n* (%)		South Nias, *n* (%)		Overall, *n* (%)
	DP	AL	DP	AL	DP	AL	
Wild-type	12/25 (48.0)	12/25 (48.0)	12/23 (52.2)	9/23 (39.1)	14/27 (51.9)	13/27 (48.1)	72/75 (96.0)
Mutant	0/25 (0)	1/25 (4.0)	0/23 (0)	2/23 (8.7)	0/27 (0)	0/27 (0)	3/75 (4.0)

7.3.2 Parasite clearance dynamics by qPCR

A total of 126 (41.7%) patients showed qPCR amplification on day 0 and these samples were then measured for parasite clearance dynamics for the first 72 hours following ACT treatment using the duplex qPCR assay. Of these, 72 samples were in the DP group and 54 in the AL group. The proportion of patients with detectable parasite DNA and relative parasite density were measured daily by qPCR as plotted in Figure 7.5. At 72 hours, 8.3% (6/72) and 11.1% (6/54) of patients in DP and AL groups harboured residual parasitaemia (*P*=0.59). These proportion detected by qPCR were significantly higher compared to those detected by microscopy (9.3% by qPCR versus 1.4% by microscopy, *P*=0.002). The median density of these residual parasites was 17.2% (of initial density 464 parasite/µL, IQR 7.0% - 70.9%) in DP and 2.4% (of initial density 304 parasite/µL, IQR 0.6% - 12.8%) in AL against pre-treatment parasite density. We did not find any risk factor including age, regency, treatment, or baseline genetic profiles including the presence of K13 mutation T474A to be associated with qPCR positivity at 72 hours (Table 7.9.). Two out of three patients with this mutation cleared parasites within 24 hours, and one patient was excluded from the study due to failure to amplify DNA by qPCR. All but one isolate with qPCR-detectable parasites on day 3 were also not detected by microscopy, and patients were only followed-up again on the next scheduled follow-up on day 7. We assumed that parasites would be cleared by day 7, therefore the mean parasite clearance times were 1.6 and 1.9 days in DP and AL groups, respectively. Mean PRR50 and PRR90 were 1.01 and 1.04 days in DP group, and 1.0 and 1.04 days in AL group.

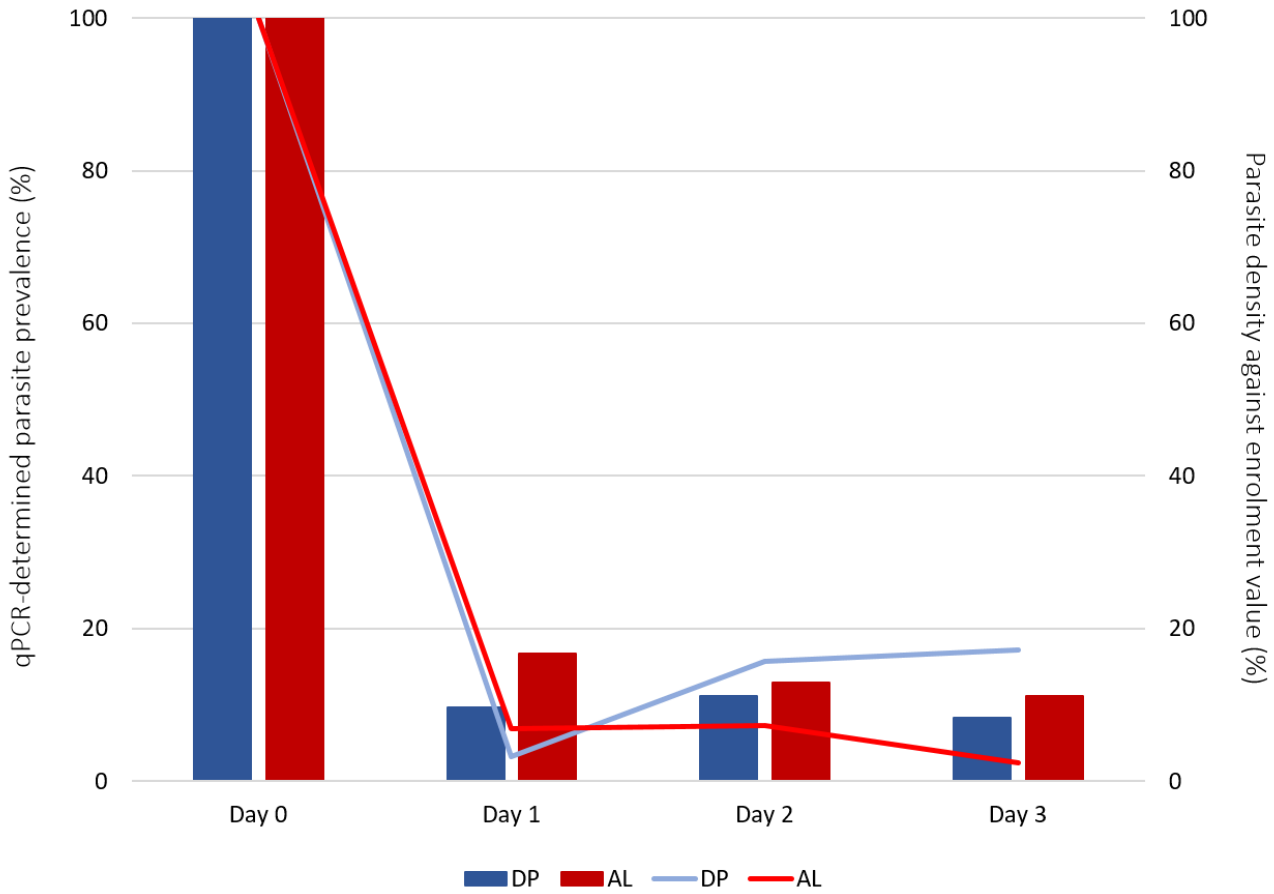


Figure 7.5. Parasite prevalence and relative parasite density as determined by qPCR from days 0 to 3. Left-y-axis, qPCR parasite prevalence (bars). Right-y-axis, median relative parasite density relative to enrollment value. (*n*=72 for DP and *n*=54 for AL).

Table 7.9. Risk factor associated with qPCR positivity at day 3

Risk Factor	*n*	Odds Ratio	95% Confidence Interval	*P* Value
Treatment				
DP	129	0.75	0.23 – 2.45	0.63
Location				
Batubara	129	Reference		
Langkat		2.0	0.19 – 21.23	0.56
South Nias		1.37	0.15 – 11.78	0.78
Age	129	0.99	0.96 – 1.03	0.84
Agegroup				
<5 years	129	Reference		
5 - <15 years		1.15	0.34 – 3.87	0.82
≥ 15 years		1		
Sex				
Male	129	0.31	0.08 – 1.19	0.07
Self-reported fever at enrollment	129	1.29	0.32 – 5.14	0.721
Log-parasite density	121	0.86	0.50 – 1.47	0.56
Pfcrt haplotype* on day 0				
CVMNK	41	1.21	0.11 – 13.25	0.88
CVIET	40	1		
SVMNT	41	0.7	0.06 – 7.78	0.77
Pfmdr1 haplotype* on day 0				
86N/184F	59	4.17	0.33 – 53.12	0.27
86Y/184Y	59	1.21	0.21 – 6.9	0.83
86N/184Y	58	0.68	0.07 – 6.34	0.73

*Odds ratio was calculated against other isolates harbouring haplotypes other than the measured haplotype

7.3.3 Treatment failures

Thirty-seven (12.8%) patients were classified as having treatment failures according to the WHO treatment outcomes in a modified intention to treat (mITT) analysis. Of those, 29 patients were parasite positive by microscopy during follow-up period and 8 withdrew from the study. Samples on day of failure from the 29 patients were screened by nested PCR for species determination. 34.7% (10/29) amplified parasite DNA (Table 7.10.) and only 10.4% (3/29) had recurrent *P. falciparum* infection. Among *P. falciparum* recurrences, only one patient was confirmed as having true recrudescence, while two had new infections. Genetic profiles of recrudescent parasite at enrolment and day of failure are shown in Table 7.11.

Interestingly, PCR revealed *P. malariae* to be the predominant species among samples with microscopy-positive treatment failures. 50% of post-treatment *P. malariae* infection appeared as single infection and 20% as mixed infection with one case occurring each at days

3, 7, 21, 35 and 42, and two cases were detected at day 14. Only 2 of these patients had *P. malariae* detected by PCR at enrolment and failed treatment at day 7 and 14.

Table 7.10. Species confirmation by PCR for patients classified for treatment failures identified by microscopy

Treatment	Patient ID	Species at enrolment by PCR	Day of failure	Species at day of failure by PCR
DP	051	*P. falciparum, P. malariae, P. knowlesi*	7	*P. malariae*
	172	*P. vivax*	14	*P. malariae*
	190	*P. falciparum, P. vivax*	7	*P. falciparum*
	208	*P. falciparum, P. knowlesi*	21	*P. malariae*
	217	*P. falciparum, P. vivax*	42	*P. malariae*
	226	Negative	14	*P. knowlesi*
	286	*P. malariae*	14	*P. malariae*
	354	*P. falciparum*	3	*P. falciparum, P. malariae*
AL	220	*P. vivax, P. knowlesi*	35	*P. falciparum, P. vivax, P. malariae*
	266	Negative	14	*P. knowlesi*

Table 7.11. Genetic profiles at baseline and follow-up days for DP-treated patient ID 190 with recrudescent *P. falciparum* parasitaemia

Sampled day	Pfcrt[a]	Pfmdr1[b]	Pfk13	Pfmdr1 CN
Day 0	SVMNT	YYD	WT	Multi copy
Day 1	SVMNT	YYD	WT	NA
Day 2	SVMNT	YYD	WT	NA
Day 3	SVMNT	YYD	WT	Multi copy
Day 7	SVMNT	YYD	WT	Single copy

[a]Codon 72-76 of *Pfcrt* gene; [b]Codon 86, 184 and 1246 of *Pfmdr1* gene; WT=Wild-type; NA=Not available

7.3.4 Subpatent *Plasmodium* recurrences detected by qPCR

qPCR detected 7 (2.6%) and 14 (5.2%) patients with subpatent *Plasmodium* spp. recurrences at day 28 and day 42, respectively. Species confirmation by nested PCR for the majority of these, 5 and 11 patients in days 28 and 42, respectively, were indeterminate, and these patients were regarded as cured in the analysis. Two patients with confirmed diagnosis at day 28 each had mixed *P. falciparum* and *P. vivax* infection, and *P. vivax* only, respectively, while at day 42, 2 patients were confirmed as having *P. vivax* infection (Table 7.12.). qPCR positivity on day 3 (OR 10.5, 95% CI 1.3 – 82.8, *P*=0.03) was associated with increased qPCR positivity at day 28.

Table 7.12. *Plasmodium* spp. recurrences at day 28 and day 42 as detected only by qPCR between two treatment arms

	qPCR positive	DP, *n* (%)	AL, *n* (%)	*P* value
18S rRNA nested PCR	Day 28			
	Negative	3/137 (2.2)	2/135 (1.5)	0.26
	P. falciparum	1/137 (0.7)	0/135 (0)	
	P. falciparum & vivax	1/137 (0.7)	0/135 (0)	
18S rRNA nested PCR	Day 42			
	Negative	5/137 (4.4)	6/135 (4.4)	0.46
	P. falciparum (%)	0/137 (0)	0/135 (0)	
	P. vivax (%)	2/137 (1.5)	0/135 (0)	

7.3.5 Subpatent *P. falciparum* recurrences detected by nested PCR of *pfmdr1*

Amplification of *pfmdr1* fragment 1 was the most sensitive tool for detection of *P. falciparum* infection, therefore to increase our ability to detect *P. falciparum* recurrences, we deployed this assay to screen our samples for recurrences. The proportion of subpatent *P. falciparum* recurrences as determined by nested-PCR targeting *pfmdr1* gene was higher during follow-up than those detected by qPCR (Figure 7.6.), however it was not statistically different between the two treatment arms (Hazard ratio 1.08; 95% CI, 0.70-1.62, *P*=0.09). There were a total of 31 and 45 treatment failures at days 28 and 42 in the DP arm, and a total of 28 and 43 treatment failures at days 28 and 42 in the AL arm, respectively (Table 7.13.). The risk of having subpatent *P. falciparum* recurrence increased in patients residing in Langkat (unadjusted HR 2.87; 95% CI, 2.29-3.60; *P*<0.001), and with starting higher parasite density (unadjusted HR 1.28, 95% CI 1.10-1.49; *P*<0.001), (Table 7.15.). However, after adjustment with other risk factors (Table 7.16.), living in Langkat was the only factor increased the risk to acquire subpatent *P. falciparum* infection at day 42 (adjusted HR 3.28; 95% CI, 2.14-4.73; *P*<0.001).

Table 7.13. Subpatent *P. falciparum* recurrences after DP and AL at day 28 and day 42

PCR recurrences	DP (*n*=139)*	AL (*n*-=142)	Hazard Ratio	Log-rank
Day 28	21.7% (95% CI 0.16 – 0.29)	19.7% (95% CI 0.14 – 0.27)	1.13 (95% CI 0.68 – 1.88), *P*=0.639	0.607
Day 42	31.5% (95% CI 0.24 – 0.39)	30.4% (95% CI 0.24 – 38.7)	1.08 (95% CI 0.70 – 1.62), *P*=0.09	0.741

*DP as the reference for treatment

Table 7.14. Proportion of *P. falciparum* recurrences according to study sites

Recurrence	Batubara, *n* (%)	Langkat, *n* (%)	South Nias, *n* (%)	Overall, *n* (%)
Day 28				
Yes	5/32 (15.6)	35/41 (85.4)	13/206 (6.3)	53/279 (19.0)
No	27/32 (84.4)	6 (14.6)	193/206 (93.7)	193/206 (81.0)
Day 42				
Yes	4/33 (12.1)	36/41 (87.8)	19/206 (9.2)	59/280 (21.1)
No	29/33 (87.9)	5/41 (87.8)	187/206 (90.8)	221/280 (78.9)

Table 7.15. Unadjusted Hazard Ratio for *Plasmodium* spp. recurrences by day 42

Risk Factor	Hazard Ratio	95% Confidence Interval	*P* Value
Treatment			
DP	1.07	0.70 – 1.62	0.09
Location	0.63	0.49 – 0.79	0.001
Location in Langkat	2.87	2.29 – 3.60	**0.001**
Age			
<5 years	1.05	0.55 – 1.98	0.87
5 - <15 years	0.84	0.67 – 1.05	0.11
≥ 15 years	1.11	0.96 – 1.28	0.14
Sex			
Male	0.82	0.54 – 1.26	0.37
Temperature > 37.5 at enrollment	0.81	0.42 – 1.57	0.54
History of malaria in previous 1 month	1.24	0.62 – 2.51	0.54
Parasite density	1.28	1.10 – 1.49	**0.001**
Presence of parasites on day 3			
By microscopy	2.30	0.56 – 9.35	0.24
By qPCR	1.50	0.59 – 3.84	0.39
Pfcrt haplotype* on day 0			
CVMNK	0.83	0.34 – 2.03	0.68
CVIET	1.15	0.35 – 3.78	0.82
SVMNT	1.18	0.51 – 2.76	0.69
Pfmdr1 haplotype* on day 0			
86N/184F	1.23	0.29 – 5.13	0.77
86Y/184Y	2.09	0.87 – 5.02	0.10
86N/184Y	0.12	0.02 – 0.86	0.03

*Hazard ratio was calculated against other isolates harbouring haplotypes other than the measured haplotype

Table 7.16. Adjusted Hazard Ratio for *Plasmodium* spp. recurrences by day 42

Risk Factor	Hazard Ratio	95% Confidence Interval	*P* Value
Treatment (DP)	1.31	0.66 – 2.60	0.45
Location in Langkat	3.28	2.14 – 4.73	**0.001**
Parasite density	1.11	0.85 – 1.46	0.45
Presence of parasites on day 3 by qPCR	1.15	0.42 – 3.15	0.78

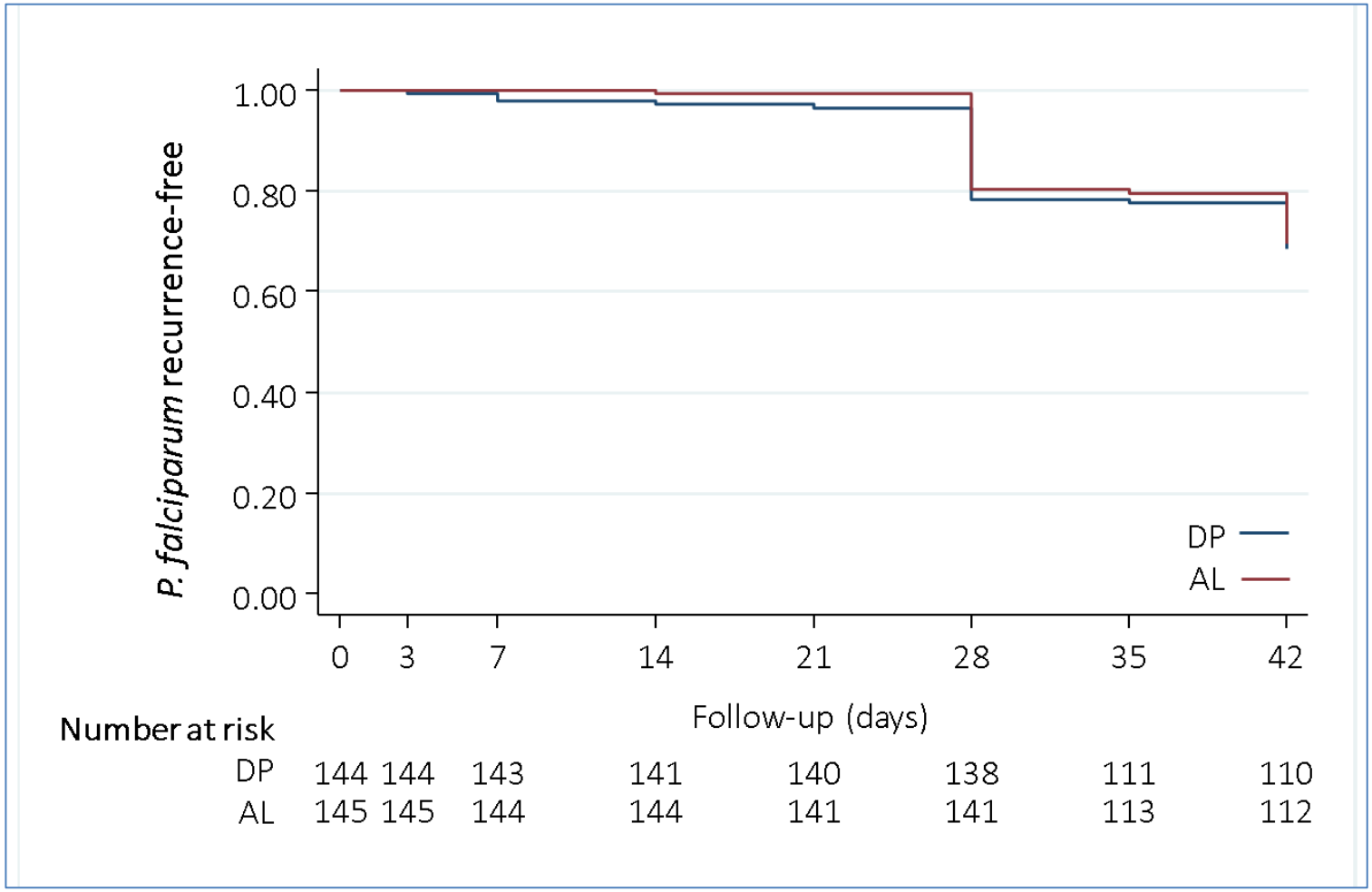


Figure 7.6. Survival rate of *P. falciparum* subpatent recurrences detected by *pfmdr1* between DP and AL at day 42 (log rank=0.741).

7.3.6 Post-treatment parasite selection

As we have provided evidence of subpatent recurrences in patients receiving DP and AL treatment, we investigated the differences in the *pfmdr1* polymorphisms between the two treatment arms before and after treatment at follow-up days 28 and 42. Genotyping data of *pfmdr1* alleles 86 and 184 from enrolment day, day 28 and day 42 were compared between both treatment arms (Table 7.17.). Similar criteria to collate the *pfmdr1* haplotype were used as in section 7.3.1. In previous studies, patients with parasites harbouring *pfmdr1* 86Y/184Y/1246Y haplotype treated with AL were significantly less likely to retain this haplotype during post-treatment follow-up, and instead AL selected parasites with *pfmdr1* 86N/184F/1246D.[273, 297] However, there are conflicting results of parasites selection after the use of DP, with some studies showed selection for the polymorphisms associated with aminoquinoline resistance[362] while others showed the opposite selection.[364] In this study, we observed a significant increase of wild-type N86 and mutant 184F at day 28 and day 42 in both treatment arms (Table 7.17.). Our findings also observed stronger selection for wild-type N86 for parasites treated with AL than that with DP at days 28 (P=0.70) and 42 (P=0.89), although they were not significant (Table 7.18). The proportion of patients carrying the haplotype NF prior to treatment were 6% and 4.3% in DP and AL, also increased to 70% and 52% in DP and AL at day 28, and to 45.4% and 56% at day 42, respectively. This post-treatment haplotype-NF replaced the dominant haplotype-YY at baseline (64.0% for DP and 76.6% for AL). Based on the study sites, we also observed increased changes to N86 in all study sites, however changes for 184F were seen in Batubara and Langkat only, but not in South Nias (Figure 7.8.).

Table 7.17. Prevalence of polymorphisms in *pfmdr1* gene before and after treatment with DP and AL in 47 individuals positive by *pfmdr1* PCR at day 28 or 42

Pfmdr1	DP, *n* (%)			AL, *n* (%)			*P* Value
	Wild-type	Mutant	Mixed	Wild-type	Mutant	Mixed	
Day 0							
N86Y	16/47 (34.0)	30/47 (63.8)	1/47 (2.1)	8/45 (21.3)	36/45* (80.0)	1/45 (2.2)	
Y184F	42/46 (91.3)	4/46 (8.7)	0/46 (0)	41/45 (91.1)	4/45 (8.9)	0/45 (0)	
Day 28							
N86Y	10/20 (50)	3/20 (15)	7/20 (35)	16/26 (61.5)	5/26 (19.2)	5/26 (19.2)	0.48
Y184F	3/20 (15)	13/20 (65)	4/20 (20)	8/25 (32)	12/25 (48)	5/25 (20)	0.39
Day 42							
N86Y	14/22 (63.6)	4/22 (18.2)	4/22 (18.2)	15/26 (57.7)	3/16 (11.5)	8/26 (30.8)	0.55
Y184F	8/22 (36.4)	11/22 (50)	3/22 (13.6)	9/25 (36.0)	13/25 (52.0)	3/25 (12.0)	0.98

*Proportion contained 2 (4.4%) samples harbouring mutant 86S.

Table 7.18. Prevalence of *pfmdr1* haplotypes after treatment with DP and AL at days 28 and 42

Pfmdr1 alleles N86 and 184F	Odds Ratio: Occurrence on day 28 versus baseline (95% CI)	Odds Ratio: Occurrence on day 42 versus baseline (95% CI)
DP		
N86/184F	OR 36.6, 95% CI 6.9-235.4, *P*=0.0001	OR 13.1, 95% CI 2.7-81.4, *P*=0.0001
AL		
N86/184F	OR 24.4, 95% CI 4.4-237.2, *P*<0.0001	OR 28.6, 95% CI 5.1-277.7, *P*<0.0001

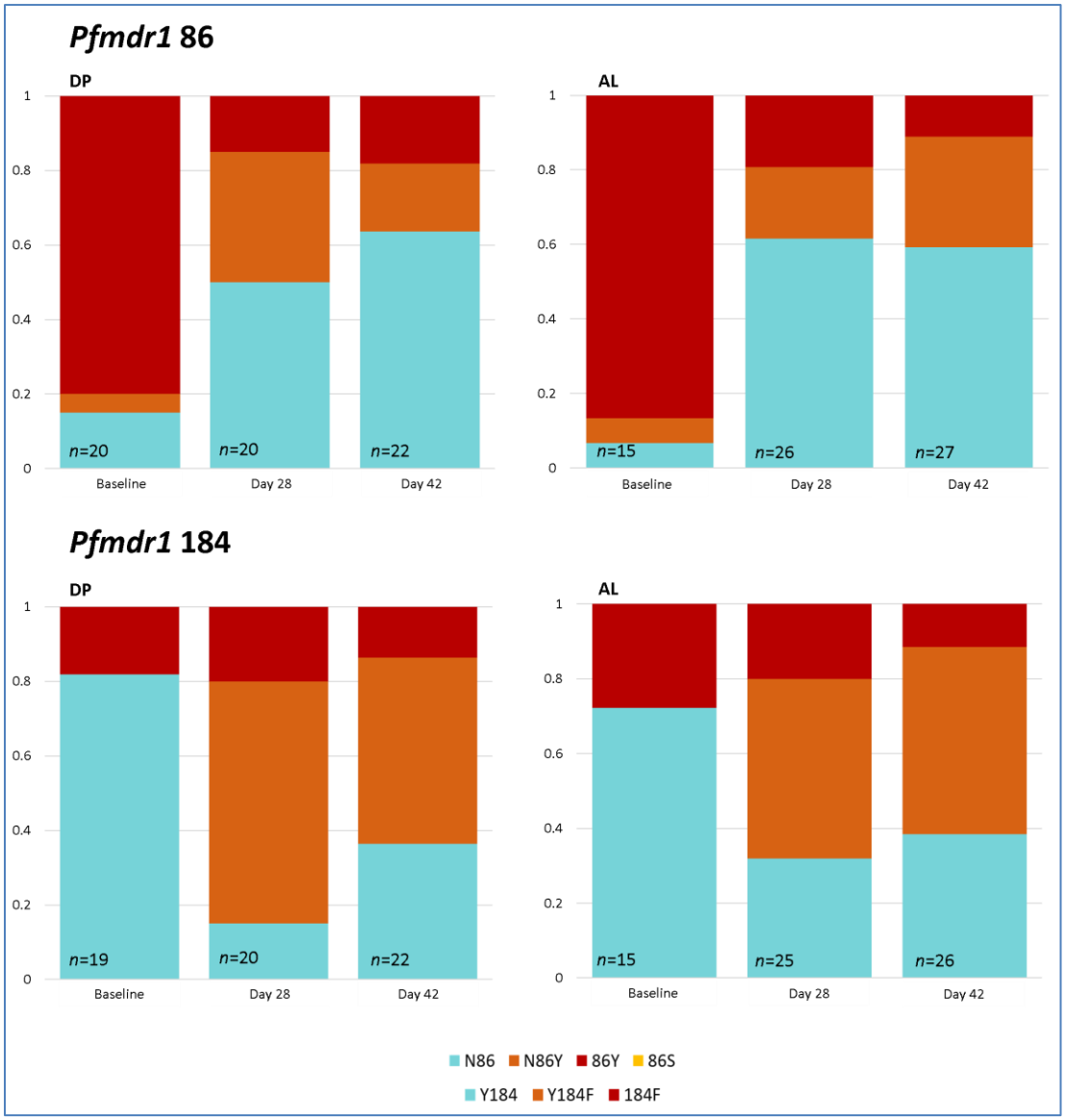


Figure 7.7. Prevalences of wild-type, mutant and mixed alleles of *pfmdr1* 86 and 184 at baseline, day 28 and day 42 in patients treated with DP and AL which harboured PCR detectable *P. falciparum* during follow-up.

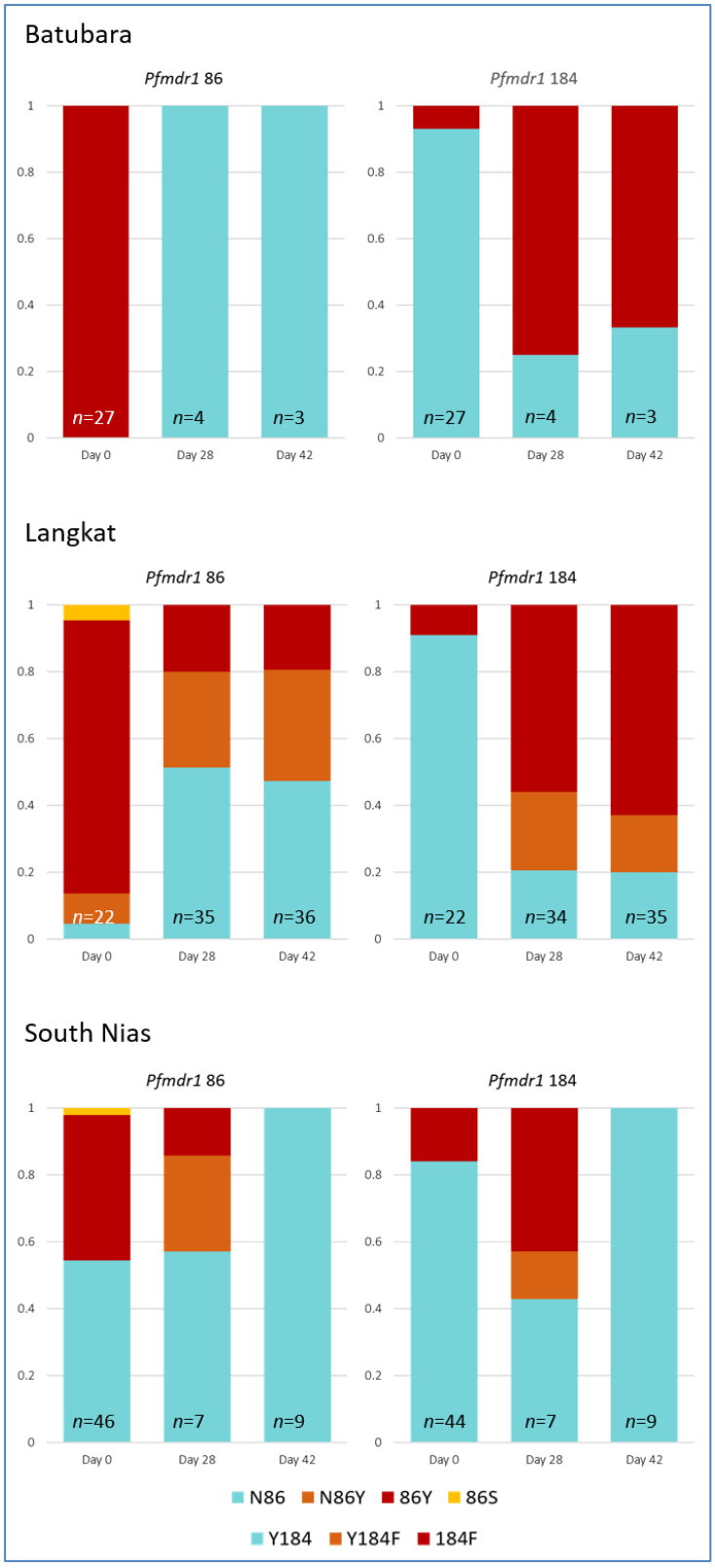


Figure 7.8. Prevalences of wild-type, mutant and mixed alleles of *pfmdr1* 86 and 184 among patients with *P. falciparum* infection according to study sites during follow-up at baseline, day 28 and day 42.

7.4 Discussion

This study investigated the responses of *P. falciparum* parasites to two ACTs, DP and AL in North Sumatera. We characterised the genetic profiles of Sumateran parasites, including key drug resistance markers including *pfcrt*, *pfmdr1* and *pfk13*, before and after treatment. We assessed parasite survival for the first 72 hours following treatment, and evaluated the occurrence of subpatent infection at days 28 and 42 by qPCR and nested PCR assays. In the rise of ACT failures in Greater Mekong sub-region, we provided important information on the efficacy status of two ACTs in western Indonesia and described the selective drug pressure on the *pfmdr1* gene given by these two tested ACTs.

Prior to treatment, the majority of Sumateran parasites carried mutant alleles of *pfcrt* and *pfmdr1*. For *pfcrt*, the SVMNT-genotype, associated with high-grade of amodiaquine resistance[181], was commonly found (73.2%), whereas in Africa and Southeast Asia CVIET-variant was the most common.[357] For *pfmdr1*, mutations in *pfmdr1* 86Y and 184Y were prevalent, of which 86Y alone or if collated with 184Y and 1246Y into haplotype is associated with resistance to chloroquine and amodiaquine.[273, 350] These characteristics of *pfcrt* and *pfmdr1* are strongly associated with amodiaquine resistance[273, 355, 359], and offer possible explanations for weaker performance of ASAQ for *P. falciparum* infection in previous studies in Indonesia.[171, 176-178]

We also provided evidence that K13 mutations were present in a small proportion of patients. Certain mutations in this *pfk13* gene propeller region have been associated with clinical artemisinin resistance, as shown by day 3 positivity after artesunate monotherapy or after 3-day treatment course of ACT.[255, 268] The polymorphisms in *pfk13* have dominated *P. falciparum* parasite populations in GMS[296, 302], whereas in this study only three (4%) patients had mutations in this gene, all carried point mutation at T474A. The mutant T474A found in our study is also different to those primarily circulated in the GMS (C580Y, F446I) or previously reported in eastern Indonesia (0.9% prevalence of C469F).[296, 302] This mutation at codon 474 has been previously reported in a small number of Cambodian isolates, however changes occurred from threonine to isoleucine and this mutant was not associated with delay in clearance.[268, 302] Patients harboring this mutant K13 parasites in this study also showed no association and treatment cleared parasites rapidly.

Genetic characterisations of *P. falciparum* populations in this study showed high frequency of wild-type *pfk13*, *pfcrt* variant of SVMNT and *pfmdr1* haplotype 86Y/184Y among

our parasites, suggesting high sensitivity of these parasites to artemisinin and lumefantrine *in vivo*[268, 273, 297], and modest sensitivity to piperaquine *in vitro*.[355] Our findings on low prevalence of day 3 positivity detected by both microscopy (1.4%) and qPCR (9.3%) also supported the expected high sensitivity of local parasites to artemisinin. Both regimens cleared parasites rapidly within 2 days after receiving the first dose, implying no immediate threat for artemisinin sensitivity in western Indonesia. However, the presence of variation at the *pfk13* locus in Sumateran parasites still reinforces the need for further surveillance of this gene. Future study needs to evaluate whether this *pfk13* T474A has a role in conferring resistance to artemisinin or will further spread at the expense of wild-type K13 parasite.

In this study, we observed high proportions of subpatent recurrences at days 28 and 42 in both treatment groups despite a suggested sensitive parasite background to the study drugs. The occurrence of submicroscopic infection, however, was not associated with qPCR-detected residual parasitaemia at day 3, different to the association previously shown in a study from Kenya.[196] Following excellent parasite clearance, proportion of submicroscopic *P. falciparum* carriages in this study were detected as high as 20% and 30% of all individuals at days 28 and 42. One plausible explanation for this event is that individuals were exposed by drug-tolerant parasites and survived the post-treatment protection given by the partner drug of ACT, and therefore they appeared to have became re-infected. We have demonstrated that our study sites in North Sumatera posed a high transmission risk of malaria (see Chapter 4), despite having a pre-elimination status by the national malaria control programmes. Thus, subjects in our study posed high risk to develop reinfection. Our observation also revealed significant changes in the *pfmdr1* polymorphisms from dominant YY prior to treatment to majority NF at both days 28 and 42 (Figure 7.7.). Parasites with the *pfmdr1* 86Y/184Y have been associated with decreased sensitivity to amodiaquine but increased sensitivity to lumefantrine, while parasites carrying alleles 86N/184F have been associated with amodiaquine-sensitive but lumefantrine-tolerance.[273, 374] Our findings supported these studies with AL effectively cleared YY-parasites but hosts were later reinfected with parasites carrying NF. On the other hand, DP is known for the long post-treatment protection among other ACTs.[203] Therefore, our findings were unexpected. Selective pressure given by DP in *Pfmdr1* gene is still unclear. Our parasites predominantly harboured the wild-type *pfmdr1* N86 during recurrent episodes. This finding is in contrast to previous studies where DP select for mutant 86Y[197, 275, 362], but consistent with others.[297, 363, 364] Moreover, our *pfmdr1* copy numbers data was too small, and we lack of information on *pfplasmepsin2* copy numbers which have

restraint our efforts to understand parasites genetic responses to the study drugs in particular for DP.

Our study shows DP and AL are still highly effective for treating uncomplicated *P. falciparum* infection. *P. falciparum* recrudescence was only confirmed in a single participant during 42 days of follow-up. By PCR, we noted *P. malariae* to be the most common cause of treatment failures among our microscopy-detected treatment failures. We have shown in our study settings that multiple malaria infections were not uncommon.[29] In Indonesia, the recommendation for any malaria infection is 3-day therapy with DP and additional primaquine depending on infecting species.[128] However, the efficacy of this combination to non-falciparum malaria has not been widely studied. Few studies have shown low density *Plasmodium* spp. infections persisted following the standard 3-day regimen of ACT.[51, 52, 375] Mixed infections in Ghanaian and Ugandan children treated with DP and AL observed less sensitivity of *P. malariae* to these drugs as shown by persistence or recurrence of *P. malariae* on follow-up days.[51, 52] One plausible explanation is that parasites were sequestered or dormant and therefore evade antimalarials activity. Another theory is that the 72-hour life-cycle of *P. malariae* gives advantage for the parasites to evade the effect of artemisinin and leaves survivors.[15, 51, 376] Further studies are needed to determine the adequate dosage and length of treatment of ACT for this particular species.

This study has several limitations. First, poor microscopy specificity was presented especially in South Nias (see Chapter 4), resulting in only one-third of our samples to be able to be genotyped for drug resistance markers. The small sample size was not ideal, but our data was sufficient to conclude the baseline genetic profiles of Sumateran parasites. Second, we have limited information on markers of piperaquine sensitivity to these parasites, as we were only successful to analyse *pfmdr1* copy number in a small numbers of isolates, and we did not perform analysis on *pfplasmepsin2* copy number. Another weakness of our study was that post-treatment genotyping was only done for *pfmdr1* gene. Amplification for *pfcrt* and *pfk13* on recurrences isolates were performed but failed to show results, this is mainly due to low density of parasites of *P. falciparum* carriages during recurrences. Last, we hypothesized that high proportion of subpatent recurrences was due to reinfection by piperaquine and/or lumefantrine resistant-parasites. Nevertheless, we did not analyse the multiplicity of infection of isolates at enrolment, thus we are not able to exclude that recurrent infections with parasites carrying wild-type genotype were due to a recrudescence event by undetected subpopulations carrying N86 prior to treatment.

Our work has important implication for the national malaria control programmes. This is the first study to report the presence of *pf*k13 mutation in an *in vivo* study from western Indonesia. We also show that DP and AL are still highly efficacious for the treatment of uncomplicated falciparum malaria, with strong evidence of low risk for artemisinin resistance. Submicroscopic infection was detected in high proportion within 4 weeks after ACT treatment. Potential contribution of these subpatent infections on post-treatment transmission is worrying and threatens the efforts for malaria elimination in this region, therefore need to be studied further. Training for microscopists is recommended to enable malaria case detection and correct identification of parasite species. Regular monitoring on the prevalence of K13 mutants and further investigations on ACT efficacy for *P. falciparum* and other *Plasmodium* species are needed to maintain the sensitivity of antimalarial drugs in western Indonesia.

Chapter 8

DISCUSSION & FUTURE DIRECTIONS

8 DISCUSSION AND FUTURE DIRECTIONS

8.1 Discussion

Malaria is still a major public health problem with 3.8 billion people at risk in 2016 as estimated by the WHO.[1] In the Southeast Asian and Western Pacific region, all countries are committed to eliminate malaria by 2030 and many of those are already in malaria elimination or pre-elimination stage.[1, 377, 378] Nevertheless, these countries, in particular those in the Greater Mekong sub-region (GMS), pose the highest risk of developing drug resistance to *P. falciparum* parasite.[185] Resistance of *P. falciparum* to the most potent antimalarial existing, artemisinin, has been described in western Cambodia in 2008.[252, 253] Since then, resistance to this drug has also been reported in other parts of Southeast Asia including Thailand, Vietnam, Myanmar, Laos and China.[120, 203, 254-259] A substantial progress in understanding of the resistance mechanisms to artemisinin and the extent of this drug resistance in Southeast Asia has been made, with most investigations performed in the area where resistant-parasites were first described, and to the countries adjacent to the epicenter of drug resistance.[185, 268, 379] These efforts were aimed to contain the spread of resistance especially westward to Africa where the highest burden of malaria is taking place.[1]

Indonesia is on the east of mainland Southeast Asia and does not share borders with the countries that are currently affected by artemisinin-resistant parasites. However, nearly 70 million of its population are at risk of acquiring malaria infection.[1] Hence, the development of *P. falciparum* resistance to antimalarial in this region would become a public health disaster, and would interrupt malaria control and elimination programmes. The work presented in this thesis adds to the knowledge base of malaria epidemiology and parasites response to ACT treatment in North Sumatera, Indonesia. The insights from this work allow greater understanding of *P. falciparum* genotypic and phenotypic responses to ACTs particularly for DP and AL, and help the national malaria control programmes to progress towards malaria elimination by providing updates on recommendation for antimalarial use in this region.

The first objective of the thesis was to investigate malaria distribution in reportedly low transmission settings in North Sumatera province, Indonesia. In chapter 4, we have described the molecular epidemiology of *Plasmodium* species in three regencies: Batubara, Langkat and South Nias, in North Sumatera province, Indonesia. We identified 4 *Plasmodium* species to be transmitted in this region including *P. falciparum*, *P. vivax*, *P. malariae*, and *P. knowlesi*. We detected a substantial numbers of infection by *P. knowlesi* (443 cases) in the population using a novel assay for detection of this species. We stress the importance on training microscopists for better identification of *Plasmodium* species now that we have shown *Plasmodium* species other than *P. falciparum* and *P. vivax* were widely distributed. We also emphasise the importance to include treatment recommendation for *P. knowlesi* in the national malaria guidelines. North Sumatera is currently aiming for malaria elimination status in 2020, but our study has shown that a substantial numbers of individuals still carried parasites. These 3 regions however could not represent the whole region of North Sumatera. These areas were particularly complex and remotely located, and they were selected because of their high burden of malaria as reported in the Provincial Health Report.[312, 316] But, with the use of molecular tools, our findings revealed a higher burden of malaria than previously reported. Furthermore, we have identified a huge proportion of submicroscopic infections contributing to overall malaria burden (71.3% of all infections). This means a substantial number of infected individuals were left undiagnosed and untreated. Although this was not studied further in this work, submicroscopic infection has been associated with higher transmission potential to mosquitoes.[188] Therefore, these results have important implication on the update of malaria status in this region, and further to help the national malaria control programmes on developing strategies for malaria control plans to achieve the goal of elimination in 2020.

We further characterised samples identified as *P. falciparum* infection by polymerase chain reaction (PCR) for drug resistance markers. Polymorphisms in the *pfcrt*, *pfmdr1* and *pfkelch13*, the markers for chloroquine, amodiaquine, mefloquine, lumefantrine and artemisinin resistance, were described in Chapter 5.[181, 268, 269, 273] We show that the haplotype SVMNT of *pfcrt* gene was prevalent in this region, different to that found in Southeast Asia and Africa.[181, 272, 357] We also show that the *pfmdr1* 86Y and 184Y were prevalent in two of our study sites (Batubara and Langkat), but not in South Nias where 86N and 184Y were dominant. Prevalence of the *pfcrt* SVMNT and *pfmdr1* 86Y/184Y as shown in our findings were similar as reported before, and these haplotypes have been reported to confer chloroquine and amodiaquine resistance.[181, 355] Therefore, as described in Chapter 5, we propose the treatment

failures following treatment with ASAQ as demonstrated in previous *in vivo* studies across Indonesia[171, 176-178] might be due to the high prevalence of the *pfcrt*-SVMNT and *pfmdr1*-YY haplotypes among *P. falciparum* isolates. Additionally, we were the first to describe mutations in the *pfkelch13* among samples from western Indonesia. One mutation at T474A was of particular interest, occurring in 3% of analysed samples. However, the impact of this mutation phenotypically has to be confirmed by *in vivo* and *in vitro* phenotypic studies.

The second objective of the thesis was to evaluate the efficacy of DP and AL for the treatment of uncomplicated *P. falciparum* malaria with a randomised controlled trial. Slow parasite clearance manifests when parasites are less sensitive to artemisinin and this phenotype has been reported in the Mekong countries.[120, 203, 254-259] In this study as described in Chapter 6, both drugs cleared parasites rapidly within two days after receiving the first dose of treatment (parasite clearance times of 1.52 and 1.45 days for DP and AL). Only a very small proportion of patients had parasites on day 3, 1.4% of samples in DP group and and 0.7% in AL group. We were also able to show that both regimens were highly effective (99.3% and 100% efficacy for DP and AL), suggesting parasites were sensitive to both of the partner drugs, piperaquine and lumefantrine. Interestingly, our evidence showed that DP was not superior to AL for treating *P. falciparum* infection, as reported before.[186, 204]

We also investigated the association between genetic polymorphisms and treatment outcomes, as further discussed in chapter 7. *P. falciparum* isolates in this study carried a specific genetic signature including mutant SVMNT of *pfcrt* haplotype and the YY *pfmdr1*haplotype. Wild-type *pfkelch13* was prevalent, and only 4% of patients carried mixed wild-type and mutant T474A. This parasite background does not seem to be associated with day 3 positivity or submicroscopic recurrences at days 28 or 42. This is in accordance to previous observations of high sensitivity of these genotypes to artemisinin, lumefantrine and piperaquine.[181, 268, 355] Our data also showed there was no difference between parasites harboring the *pfkelch13* mutant T474A and wild-type parasites in the parasite clearance dynamics, implying that point mutation at this codon does not modulate artemisinin susceptibility. Using a highly sensitive quantitative polymerase chain reaction (qPCR), the proportion of day 3 positivity were at 8.3% in DP and 11.1% in AL groups. Despite some proportion of submicroscopic recurrences at day 42 after both treatment DP (31.5%) and AL (30.4%), these findings were not associated with baseline genotypic profiles, day 3 positivity or parasite density at enrolment. The only factor strongly associated with day 42 recurrences was residency in Langkat.

This study also described parasite selection after treatment with DP and AL, as described in Chapter 7. Both regimens exerted selection for parasites harbouring wild-type *pfmdr1*-86, as shown with increased proportion of parasites carrying *pfmdr1* NF at days 28 and 42 compared to baseline proportion. Based on this evidence, we suggested that DP and AL were still highly efficacious for treating *P. falciparum* infection in particular for parasites with a background of *pfcrt* SVMNT and *pfmdr1* YY. But neither of the drugs provided adequate post-treatment protection to completely prevent the occurrence of submicrosopic infections by parasites carrying the *pfdmr1* NF genotypes. Given the interest of these findings, it is important to continue to monitor the efficacy of both drugs and to evaluate the selective pressure given by these drugs as manifests by changes in the genotypic profiles of *P. falciparum* parasites.

Furthermore, our findings also revealed that recurrence with *P. malariae* was not uncommon, and this species presented as the most common cause of microscopically patent recurrences in the study. Previous studies have proposed the ability of this species to evade short exposure from artemisinin which leaving survivors after treatment.[15, 51, 376] However, this hypothesis needs further confirmation and there is a need to further investigate the efficacy of ACTs for this species, more importantly to determine the accurate dosing and length of treatment of ACT for *P. malariae* infection.

Despite the several strengths of this work mentioned in each results chapter, this thesis is subject to several limitations. First, specificity of microscopy was noticeably poor in South Nias, as shown by PCR failure to amplify samples detected as positive by microscopy. PCR confirmation is not the standard methods for therapeutic efficacy studies and it is not requested (yet) in the WHO guidelines.[172, 323] However, in attempts to evaluate the association between baseline genetic polymorphisms and treatment outcomes as part of our objectives, we performed PCR amplifications on our collected samples. Due to poor microscopy, we then observed a reduced number of true *P. falciparum* isolates evaluated in the trial. The trial with 300 participants was powered to detect 5% superiority in subjects treated with DP. The reduced numbers of evaluated samples then may have resulted in the lack of statistical power to detect differences. Second, as described in Chapter 4, our cross-sectional study did not deploy a systematic sampling procedure and may have introduced bias. We previously had not worked in these 3 regions, and methods were adapted to reflect the facilities available and particular local challenges. Therefore, sampling procedures were not standardized, and were therefore not ideal for an epidemiological study and may not have provided accurate

estimation on parasite prevalence in each of the study sites. Third, more than one-third of PCR-confirmed *P. falciparum* isolates failed to subsequently amplify DNA for genotyping. This may be caused by low density of parasites as explained by large proportion of submicroscopic infections among our PCR positive samples, and reflects different levels of sensitivity in our various PCR amplification protocols. Moreover, we also faced challenges in sample storage. The settings in the three study sites were difficult and particularly for South Nias, sample collection included frequent sea journeys in small boats which may have prevented samples from being dried and stored appropriately following samplings. This could have caused poor quality of DNA in the filter papers. Fourth, we have identified mutations in the *pfkelch13* among our samples, but these findings were not reproducible during several further attempts for PCR amplifications. Therefore, our findings, although potentially important, offer only weak to moderate evidence of the presence of these variants. Furthermore, we also did not provide information on other important drug resistance markers. Analysis on *pfmdr1* copy numbers were only available in small numbers of cases, and we did not perform analysis on *pfplasmepsin2* copy numbers. Both markers have been strongly associated with the sensitivity of ACTs partner drugs including mefloquine and piperaquine. By the time we started our laboratory analysis for the trial, we had used most of our DNA for the purpose of molecular detection in our epidemiological work. The limited time also restrained us from further experiments with the remaining DNA. Further analysis on the drug resistance markers from recurrences isolates would have also added important values in our study. But, our efforts to amplify DNA for *pfcrt* and *pfkelch13* genotyping were not successful in recurrent infections and these could be contributed by the low density parasites detected in the cases. An additional limitation is that we did not measure piperaquine and lumefantrine drug levels following treatment. The aforementioned investigation would provide us with further understanding on DP and AL post-treatment prophylaxis for subpatent recurrences.

8.2 Future directions

This thesis described above extends our understanding of molecular epidemiology of malaria in North Sumatera and *P. falciparum* parasites responses to DP and AL treatment in this region. We have described the presence of four *Plasmodium* species in North Sumatera, and human *P. knowlesi* infection was not uncommon. We have also successfully shown that both DP and AL are still highly efficacious for treating uncomplicated *P. falciparum* infection,

but subsequent submicroscopic recurrences were frequent. Nevertheless, our findings raise further questions, and with awareness of challenges that lie ahead of the complicated dynamics of Sumateran parasites, the following future studies can be deployed to answer those questions:

- Case-control study to investigate human risk to acquire *P. knowlesi* infection and further to evaluate the virulence of this species in the presence of other *Plasmodium* species in this region. This is necessary to recognise which group is at the highest risk of infection thus interventions to mitigate the risk could be targeted at the right population, and to investigate within-host dynamics in the presence of multi-species infections and the contribution of this infection to cause symptomatic clinical malaria;
- Epidemiological survey to determine the true prevalence of all *Plasmodium* species in this region, the contribution of submicroscopic infections, and the role of submicroscopic infection in further transmission to mosquitoes. A strong surveillance system is important to measure progress to elimination. Consideration of circulating species when designing malaria control and elimination strategies is crucial to help in achieving malaria elimination status;
- *In vitro* study to assess *P. falciparum* and *P. knowlesi* (if not possible yet to include *P. vivax* and *P. malariae*) susceptibility to a panel of antimalarial drugs. This is especially important for a region like North Sumatera where multi-species of malaria are common. Therefore recommendation for the use of an antimalarial that is sensitive to all *Plasmodium* species can be given and adopted into the national policy for treatment of malaria;
- Evaluation on the *pfmdr1* copy numbers, *pfplasmepsin2* copy numbers, and the polymorphisms of other potentially important genes among *P. falciparum* isolates. This should be done in a regular manner to allow early changes detection in the genotypic profiles;
- Regular *in vivo* drug efficacy studies evaluating the current national recommendation, DP, in comparison with other ACTs. In this context, comparison of DP with AS-MQ will provide baseline information on the efficacy of AS-MQ and offer an alternative treatment if DP starts to fail.

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9 REFERENCES

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APPENDICES

Appendix 1. Ethical approval 1

London School of Hygiene & Tropical Medicine
Keppel Street, London WC1E 7HT
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LONDON SCHOOL of HYGIENE &TROPICAL MEDICINE

Observational / Interventions Research Ethics Committee

Miss Inke Lubis
Research Degree Student
ITD
LSHTM

14 October 2014

Dear Miss Lubis,

Study Title:Clinical efficacy of artemisinin-based combination therapy for treatment of uncomplicated *Plasmodium falciparum* malaria in North Sumatera, Indonesia and the association of molecular markers with treatment outcomes

LSHTM Ethics Ref: 8504

Thank you for your letter of 8 October 2014, responding to the Interventions Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

Approval is dependent on local ethical approval having been received, where relevant.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document Type	File Name	Date	Version
Protocol / Proposal	Case Screening Form Clinical Trial.docx	04/07/2014	1
Protocol / Proposal	Protocol for Sumatera clinical study_v2.pdf	12/09/2014	2
Protocol / Proposal	Case Record Form Clinical Trial DHP vs AL_v2.docx	12/09/2014	2
Protocol / Proposal	Case Record Form – Summary DHP vs AL_v2.docx	12/09/2014	2
Protocol / Proposal	AE report form DHP vs AL_v2.docx	12/09/2014	2
Protocol / Proposal	SAE report form DHP vs AL_v2.docx	12/09/2014	2
Information Sheet	PIS DHA-PQ vs AL_v2.docx	12/09/2014	2
Information Sheet	Informed Consent DHP vs AL_v2.docx	12/09/2014	2
Information Sheet	PIS Indonesia DHP vs AL_v2.docx	12/09/2014	2
Information Sheet	Informed Consent Indonesian DHP vs AL_v2.docx	12/09/2014	2
Protocol / Proposal	blood spot protocol.docx	07/10/2014	1

After ethical review

Any subsequent changes to the application must be submitted to the Committee via an Amendment form on the ethics online applications website. The Principal Investigator is reminded that all studies are also required to notify the ethics committee of any serious adverse events which occur during the project via an Adverse Event form on the ethics online applications website. An annual report form is required on the anniversary of the approval of the study and should be submitted during the lifetime of the study on the ethics online applications website. At the end of the study, please notify the committee via an End of Study form on the ethics online applications website. Ethics online applications website link: http://leo.lshtm.ac.uk

Yours sincerely,

[redacted]

Professor John DH Porter
Chair

ethics@lshtm.ac.uk
http://www.lshtm.ac.uk/ethics/

Improving health worldwide

Page 1 of 1

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LONDON SCHOOL of HYGIENE &TROPICAL MEDICINE

Observational / Interventions Research Ethics Committee

Colin Sutherland
II / ITD
LSHTM

24 January 2014

Dear Dr. Sutherland,

Submission Title: **Cross-sectional survey of drug resistance markers in malaria parasites from Sumatera**

LSHTM Ethics Ref: **7089**

Thank you for your response of 15 January 2014, responding to the Observational Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

Approval is dependent on local ethical approval for the amendment having been received, where relevant.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document Type	File Name	Date	Version
Information Sheet	lshtm_consent_template.docx	8/11/2013	1
Information Sheet	lshtm_pis_template-active surv v2.docx	21/11/2013	2
Information Sheet	lshtm_pis_template-passive surv v2.docx	21/11/2013	2
Protocol / Proposal	Proposal version 3.pdf	21/11/2013	3
Protocol / Proposal	CRF.docx	21/11/2013	2

After ethical review

Any subsequent changes to the application must be submitted to the Committee via an Amendment form onthe ethics online applications website. The Principal Investigator is reminded that all studies are also required to notify the ethics committee of any serious adverse events which occur during the project via anAdverse Event form on the ethics online applications website. At the end of the study, please notify the committee via anEnd of Study form onthe ethics online applications website.

Yours sincerely,

[redacted]

Professor John DH Porter
Chair

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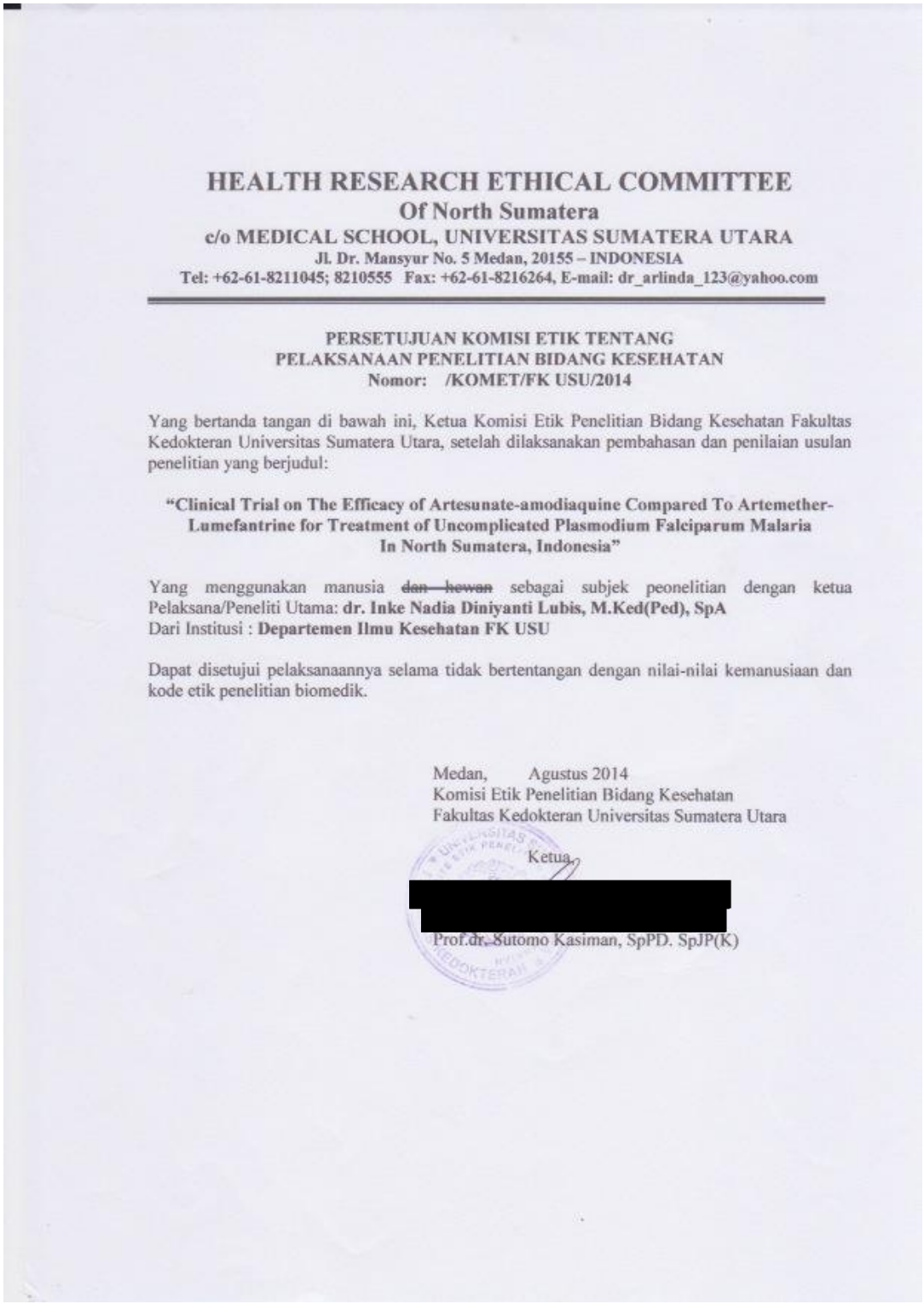
Improving health worldwide

Appendix 2. Ethical approval 2



HEALTH RESEARCH ETHICAL COMMITTEE
Of North Sumatera
c/o MEDICAL SCHOOL, UNIVERSITAS SUMATERA UTARA
Jl. Dr. Mansyur No. 5 Medan, 20155 – INDONESIA
Tel: +62-61-8211045; 8210555 Fax: +62-61-8216264, E-mail: dr_arlinda_123@yahoo.com

PERSETUJUAN KOMISI ETIK TENTANG
PELAKSANAAN PENELITIAN BIDANG KESEHATAN
Nomor: /KOMET/FK USU/2014

Yang bertanda tangan di bawah ini, Ketua Komisi Etik Penelitian Bidang Kesehatan Fakultas Kedokteran Universitas Sumatera Utara, setelah dilaksanakan pembahasan dan penilaian usulan penelitian yang berjudul:

"Clinical Trial on The Efficacy of Artesunate-amodiaquine Compared To Artemether-Lumefantrine for Treatment of Uncomplicated Plasmodium Falciparum Malaria In North Sumatera, Indonesia"

Yang menggunakan manusia ~~dan hewan~~ sebagai subjek peonelitian dengan ketua Pelaksana/Peneliti Utama: **dr. Inke Nadia Diniyanti Lubis, M.Ked(Ped), SpA**
Dari Institusi : **Departemen Ilmu Kesehatan FK USU**

Dapat disetujui pelaksanaannya selama tidak bertentangan dengan nilai-nilai kemanusiaan dan kode etik penelitian biomedik.

Medan, Agustus 2014
Komisi Etik Penelitian Bidang Kesehatan
Fakultas Kedokteran Universitas Sumatera Utara

Ketua,

[redacted]

Prof.dr. Sutomo Kasiman, SpPD. SpJP(K)

Appendix 3. Case screening form

Case Screening Form – A Day 0

Case Screening Form	
Health centre name: Village/Town: Regency:	Patient identity number: Date:
Patient initial:	Patient father/husband name:
Demographic Data	
Age: Date of birth (dd/mmm/yyyy):	Sex: M ☐ F ☐
Weight: kg	Height: cm
Patient address: Contact number:	Socioeconomic status: Education level: Job: Father's job if under 20 years:
Staff name:	Sign:

Case Screening Form – B Day 0

Pre-Treatment Assessment			
Patient identity number: Age: Sex: M ☐ F ☐		Place of assessment: Study site ☐ Home ☐	Date:
Symptoms	Yes / No	Scale	Duration
Fever	Yes ☐ No ☐		
Headache	Yes ☐ No ☐		
Body ache	Yes ☐ No ☐		
Vomit	Yes ☐ No ☐		
Nausea	Yes ☐ No ☐		
Diarrhea	Yes ☐ No ☐		
Rigors/chill	Yes ☐ No ☐		
Anemia	Yes ☐ No ☐		
Cyanosis	Yes ☐ No ☐		
Convulsion	Yes ☐ No ☐		
Dark colored urine	Yes ☐ No ☐		
Skin disorder	Yes ☐ No ☐		
Use of antimalarial in the past	< 1 month: Yes ☐ No ☐	1-2 months: Yes ☐ No ☐	> 2 months Yes ☐ No ☐
If yes, please specify: *- Name of antimalarial* *- Diagnosis of malaria was made by:*	Doctor ☐ Midwive ☐ Traditional healer ☐ Self ☐ Other ☐	Doctor ☐ Midwive ☐ Traditional healer ☐ Self ☐ Other ☐	Doctor ☐ Midwive ☐ Traditional healer ☐ Self ☐ Other ☐
Use of malaria prevention	Insecticide treated nets ☐ Repellent ☐ Others: ….. ☐	Insecticide spraying ☐ Larvacide on stagnant water ☐	
Pre-Treatment Examination			
Body weight: kg Height: cm		Temperature: °C Pulse: times/min Respiratory rate: times/min Blood pressure: mmHg Liver enlargement: Yes ☐ No ☐ Spleen enlargement : Yes ☐ No ☐	

Laboratory

Thick and thin blood smears:
Species: *P. falciparum* ☐ *P. vivax* ☐ *P. malariae* ☐ *P. ovale* ☐

Were species other than P. falciparum present? Yes ☐ No ☐ (**If yes, patient is not eligible)**

Parasite density: /μL (**If parasite density < 250 μL, patient is not eligible)**

Presence of *P. falciparum* gametocyte: Yes ☐ No ☐

Rapid diagnostic test: *P. falciparum* Yes ☐ Mixed infection Yes ☐ Other *Plasmodium* infection Yes ☐ Negative Yes ☐ **(If other species than P. falciparum is present, patient is not eligible)**	Filter paper taken: Yes ☐ No ☐ Pregnancy: Yes ☐ No ☐ **(If yes, patient is not eligible)** Hemoglobin: gr/dL

Inclusion Criteria

- Male or female;
- All patients over 6 months of age;
- Fever as defined by axillary temperature ≥ 37.5 °C or history of fever during the 48 hours before recruitment;
- Infection with *P. falciparum* detected by microscopy;
- Parasitaemia >250 /μl blood;
- Ability to swallow oral medication;
- Ability and willingness to comply with the protocol for the duration of the study and to comply with the study visit schedule;
- Informed consent from the patient of from a parent or guardian in the case of children;
- Absence of history to hypersensitivity reactions or contraindication to antimalarial drugs;
- Not currently consuming antibiotic with antimalarial activity (such as cotrimoxazole, macrolides, tetracycline or doxycycline) (Appendix 5)

Does the patient meet all inclusion criteria? Yes ☐ No ☐ (**If no, patient is not eligible)**

Exclusion Criteria

- Presence of *P. vivax* infection detected by microscopy;
- Presence of general danger signs in children under 5 years or signs of severe falciparum malaria according to the definitions of WHO (2000) (Appendix 6);
- Presence of severe malnutrition according to WHO child growth standards (Appendix 7);
- Presence of febrile conditions caused by diseases other than malaria;
- Presence of severe anemia (Hemoglobin < 7 gr/dl);
- Received any of the study drugs within the past 4 weeks;
- Received any antimalarial within the last 2 weeks;
- Recurrent vomiting (necessitating more than a single repeat dose);
- Pregnant (demonstrated by positive result of β-HCG in women of childbearing age);
- Lactating mother

Does the patient meet any of the exclusion criteria? Yes ☐ No ☐
(If yes, patient is not eligible)

If yes, please specify the reason for exclusion:

Patient Informed Consent and Assent	
Consent form signed Yes ☐ No ☐ Assent form signed Yes ☐ No ☐	Patient identity number: Date (dd/mmm/yyyy):

Appendix 4. Case record form

Case Record Form – A Follow-Up Day 0

Case Record Form	
Health centre name: Village/Town: Regency:	Patient identity number: Date:
Patient initial:	Patient father/husband name:
Demographic Data	
Age: Date of birth (dd/mmm/yyyy):	Sex: M ☐ F ☐
Weight: kg	Height: cm
Patient address: Contact number:	Socioeconomic status: Education level: Job: Father's job if under 20 years:
Randomised treatment allocation:	Arm 1: DHA-P ☐ Arm 2: AL ☐
Outcome of trial:	ACPR: ☐ ETF: ☐ LCF: ☐ LPF: ☐ Withdrawn: ☐ Lost to follow-up : ☐
Staff name:	Sign:

Pre-Treatment Assessment

Patient identity number: Age: Sex: M ☐ F ☐		Place of assessment: Study site ☐ Home ☐	Date:
Symptoms	Yes / No	Scale	Duration
Fever	Yes ☐ No ☐		
Headache	Yes ☐ No ☐		
Body ache	Yes ☐ No ☐		
Vomit	Yes ☐ No ☐		
Nausea	Yes ☐ No ☐		
Diarrhea	Yes ☐ No ☐		
Rigors/chill	Yes ☐ No ☐		
Anemia	Yes ☐ No ☐		
Cyanosis	Yes ☐ No ☐		
Convulsion	Yes ☐ No ☐		
Dark colored urine	Yes ☐ No ☐		
Skin disorder	Yes ☐ No ☐		
Other complaints:			

Pre-Treatment Examination

Body weight:	kg	Temperature:	°C
Height:	cm	Pulse:	times/min
		Respiratory rate:	times/min
		Blood pressure:	mmHg
		Liver enlargement:	Yes ☐ No ☐
		Spleen enlargement :	Yes ☐ No ☐

Laboratory

Thick and thin blood smears:
Species: *P. falciparum* ☐ *P. vivax* ☐ *P. malariae* ☐ *P. ovale* ☐

Were species other than P. falciparum present? Yes ☐ No ☐ **(If yes, patient is not eligible)**

Parasite density: /µL **(If parasite density < 250 µL, patient is not eligible)**

Presence of *P. falciparum* gametocyte: Yes ☐ No ☐

Rapid diagnostic test: *P. falciparum* Yes ☐ Mixed infection Yes ☐ Other *Plasmodium* infection Yes ☐ Negative Yes ☐ **(If other species than P. falciparum is present, patient is not eligible)**	Filter paper taken: Yes ☐ No ☐ Pregnancy: Yes ☐ No ☐ **(If yes, patient is not eligible)** Hemoglobin: gr/dL

Medication Administration

Dihydroartemisinin Piperaquine ☐			Artemether Lumefantrine ☐	
Name of antimalarial	Time of dose (hh:mm)	Number of tablets	Vomit	Time (hh:mm)
			Yes ☐ No ☐	
			Yes ☐ No ☐	
			Yes ☐ No ☐	
			Yes ☐ No ☐	
Other medication				
			Yes ☐ No ☐	
			Yes ☐ No ☐	

Assessment

Patient identity number: Age: Sex: M ☐ F ☐		Place of assessment: Study site ☐ Home ☐	Date:
Symptoms	Yes / No	Scale	Duration
Fever	Yes ☐ No ☐		
Headache	Yes ☐ No ☐		
Body ache	Yes ☐ No ☐		
Vomit	Yes ☐ No ☐		
Nausea	Yes ☐ No ☐		
Diarrhea	Yes ☐ No ☐		
Rigors/chill	Yes ☐ No ☐		
Anemia	Yes ☐ No ☐		
Cyanosis	Yes ☐ No ☐		
Convulsion	Yes ☐ No ☐		
Dark colored urine	Yes ☐ No ☐		
Skin disorder	Yes ☐ No ☐		
Other complaints:			

Examination

Body weight:	kg	Temperature:	°C
Height:	cm	Pulse:	times/min
		Respiratory rate:	times/min
		Blood pressure:	mmHg

Laboratory

Thick and thin blood smears:
Presence of asexual *P. falciparum* parasite: Yes ☐ No ☐

Parasite density: /µL

Presence of *P. falciparum* gametocyte: Yes ☐ No ☐

Were species other than P. falciparum present? Yes ☐ No ☐ **(If yes, patient is not eligible)**
If yes, which species: *P. vivax* ☐ *P. malariae* ☐ *P. ovale* ☐

Filter paper taken: Yes ☐ No ☐
Filter paper taken for drug concentration: Yes ☐ No ☐

Adverse Events				
Presence of an adverse event: Yes ☐ No ☐ If yes, name the adverse event: Is it a serious adverse event? Yes ☐ No ☐				
Medication Administration				
Dihydroartemisinin Piperaquine ☐		Artemether Lumefantrine ☐		
Name of antimalarial	Time of dose (hh:mm)	Number of tablets	Vomit	Time (hh:mm)
			Yes ☐ No ☐	
			Yes ☐ No ☐	
			Yes ☐ No ☐	
			Yes ☐ No ☐	
Other medication				
			Yes ☐ No ☐	
			Yes ☐ No ☐	

If patient is not treated

Loss to follow up	Date of patient loss	Day of study/visit
Yes ☐ No ☐		
Reason of loss to follow-up:		
Staff name:	Sign:	Date:

Appendix 5. Medications with antimalarial activity[103]

- Chloroquine, amodiaquine;
- Quine, quinidine;
- Mefloquine, halofantrine, lumefantrine;
- Artemisinin and its derivatives (artemether, arteether, artesunate, dihydroartemisinin);
- Proguanil, chlorproguanil, pyrimethamine;
- Sulfadoxine, sulfalene, sulfamethoxazole, dapsone;
- Primaquine;
- Atovaquone;
- Antibiotics: tetracycline*, doxycycline, erythromycin, azithromycin, clindamycin, rifampicin, trimethoprim;
- Pentamidin

*Tetracycline eye ointments can be used

Appendix 6. Definition of severe falciparum malaria[380]

Severe manifestation of P. falciparum malaria in adults and children

Clinical manifestation:

- Prostration
- Impaired consciousness
- Respiratory distress (metabolic acidosis)
- Multiple convulsions
- Circulatory collapse
- Pulmonary edema (radiological)
- Abnormal bleeding
- Jaundice
- Haemoglobinurea

Laboratory findings

- Severe anemia (hemoglobin < 5 gr/dL, hematorcrit < 15%)
- Hypoglycemia (blood glucose < 2.2 mmol/L or 40 mg/dL)
- Acidosis (plasma bicarbonate < 15 mmol/L)
- Hyperlactatemia (venous lactic acid > 5 mmol/L)
- Hyperparasitaemia (>4% in non-immune patients)
- Renal impairment (serum creatinine above normal range for age)

Classification of severe malaria in children

Group 1: children at increased risk for death

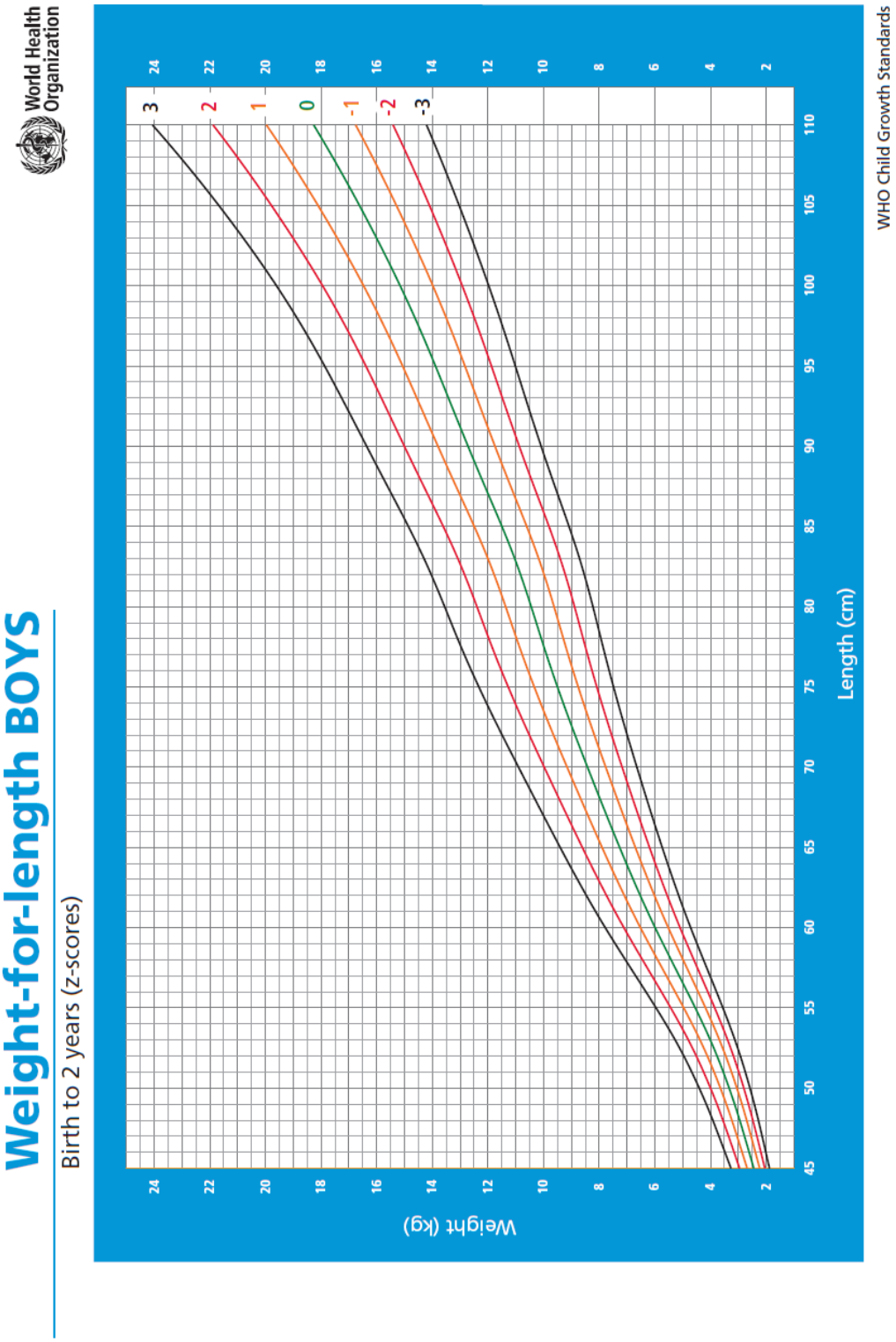
- Prostration
- Respiratory distress

Group 2: children at risk for clinical deterioration

- Hemoglobin < 5 g/dL, hematocrit < 15%)
- Two or more convulsions within 24 h

Group 3: children with persisting vomiting

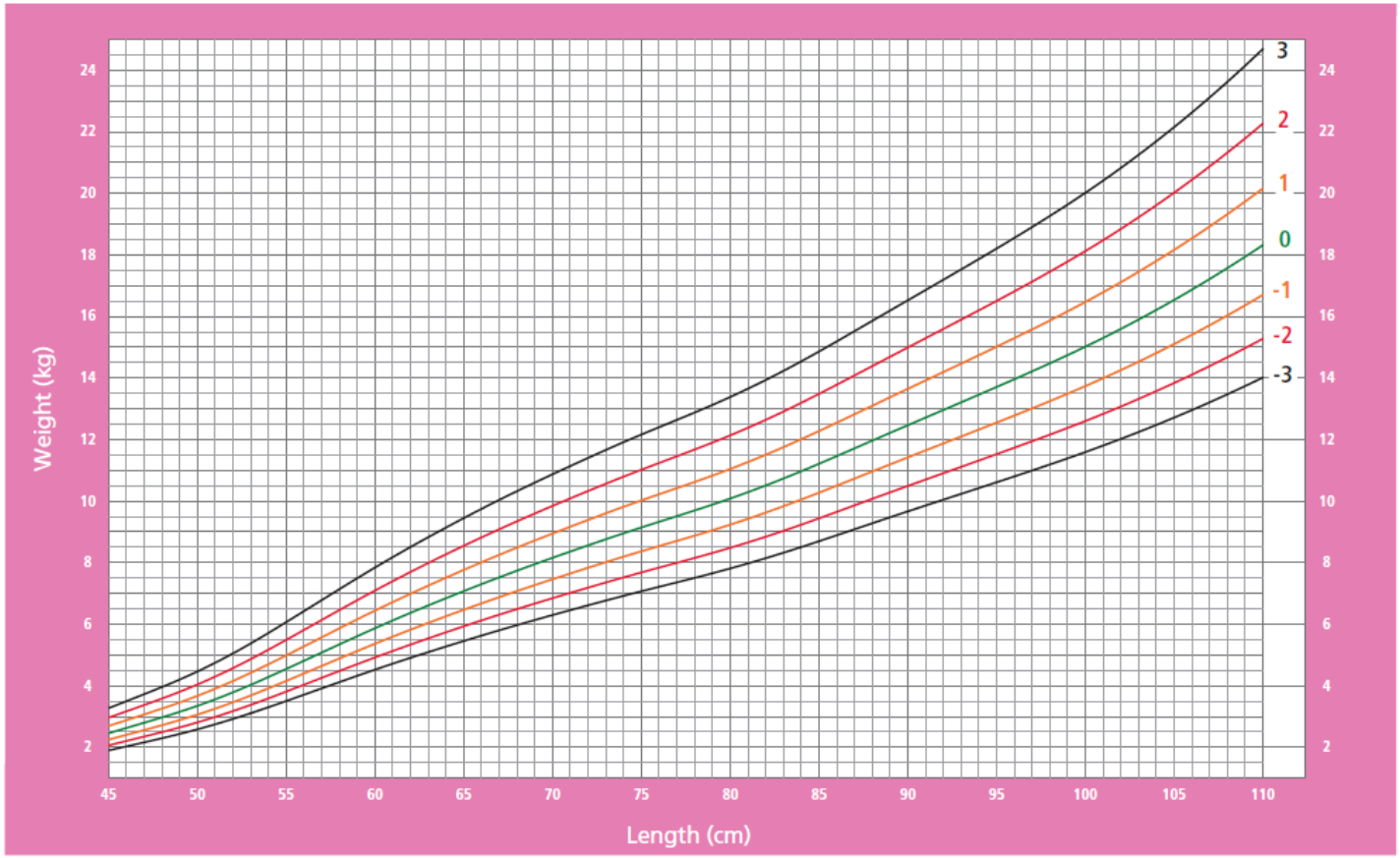
Appendix 7. WHO child growth standards[90]



Weight-for-length GIRLS

Birth to 2 years (z-scores)

World Health Organization



WHO Child Growth Standards

Appendix 8. Summary of case record form

Case Record Form – Summary

Study Site	Patient address:	Patient identity number:
Patient initial:	Date of birth (dd/mmm/yyyy): Age:	Sex: M ☐ F ☐ Weight: kg
Job: Education: Socioeconomic status:	Treatment: - DP ☐ - AL ☐	Outcome of trial: ACPR ☐ LPF ☐ ETF ☐ Withdrawn ☐ LCF ☐ Loss to follow up ☐

Visits	0	1	2	3	7	14	21	28	35	42
Date										
Danger signs (Yes/No)										
Temperature (°C)										
Presence of asexual parasite										
Parasite density (μL)										
Gametocyte (Yes/No)										
Hemoglobin (gr/dL)										
Filter paper taken (Yes/No)										
Treatment (DP / AL)										
Concomitant medication (Yes/No)										
Adverse events (Yes/No)										
Serious adverse events (Yes/No)										

Appendix 9. Adverse event report form

Adverse Event Report Form

Case Record Form	
Health centre name: Village/Town: Regency:	Patient identity number: Date: Follow-up day:
Patient initial:	Patient father/husband name:
Demographic Data	
Age: Date of birth (dd/mmm/yyyy):	Sex: M ☐ F ☐
Weight: kg	Height: cm
Patient address: Contact number:	Socioeconomic status: Education level: Job: Father's job if under 20 years:
Randomised treatment allocation:	Arm 1: DP ☐ Arm 2: AL ☐

Adverse Event		
Type of event: ☐ Anorexia ☐ Headache ☐ Dizziness ☐ Cold ☐ Cough ☐ Flu	☐ Bronchitis ☐ Rhinitis ☐ Shivering ☐ Sore throat ☐ Nausea ☐ Vomiting	☐ Abdominal pain ☐ Diarrhea ☐ Pruritus ☐ Asthenia ☐ Fever
Describe the duration and severity of adverse event:		
Describe how the reaction was treated:		

Medicines **(List the medicine suspected of causing the adverse event as well as all concomitant medicines)**					
Brand name, batch number, manufacturer name	Daily dose	Route	Start date	End date	Indications for use

Outcome:

- ☐ Recovered completely
- ☐ Not yet recovered
- ☐ Recovered with long-term consequences

If patient recovered, provide date of recovery (dd/mmm/yyyy):

Reporting Staff

Name: Qualification: Address: Phone: Email:	Date: Sign:

Appendix 10. Serious adverse event report form

Serious Adverse Event Report Form

Case Record Form	
Health centre name: Village/Town: Regency:	Patient identity number: Date: Follow-up day:
Patient initial:	Patient father/husband name:
Demographic Data	
Age: Date of birth (dd/mmm/yyyy):	Sex: M ☐ F ☐
Weight: kg	Height: cm
Patient address: Contact number:	Socioeconomic status: Education level: Job: Father's job if under 20 years:
Randomised treatment allocation:	Arm 1: DP ☐ Arm 2: AL ☐
Serious Adverse Event	
Type of event: ☐ Death ☐ Life-threatening ☐ Hospitalization or prolongation of hospitalization ☐ Permanent disability ☐ Congenital anomaly or birth defect Date of occurrence (dd/mmm/yyyy):	
Describe the serious adverse event (include all relevant laboratory results):	
Describe how the reaction was treated:	

Medicines
(List the medicine suspected of causing the serious adverse event as well as all concomitant medicines)

Brand name, batch number, manufacturer name	Daily dose	Route	Start date	End date	Indications for use

Outcome:

- ☐ Recovered completely
- ☐ Not yet recovered
- ☐ Recovered with long-term consequences

If patient recovered, provide date of recovery (dd/mmm/yyyy):

Reporting Staff

Name: Qualification: Address: Phone: Email:	Date: Sign:

Appendix 11. Participant information sheet

Information for Participants

Study title:
CLINICAL EFFICACY OF ARTEMISININ-BASED COMBINATION THERAPY FOR TREATMENT OF UNCOMPLICATED *PLASMODIUM FALCIPARUM* MALARIA IN NORTH SUMATERA, INDONESIA AND THE ASSOCIATION OF MOLECULAR MARKERS WITH TREATMENT OUTCOMES

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and to talk to others about the study, if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

1. What is the purpose of the study?

Malaria is an infectious disease that is transmitted by *Anopheles* mosquito, and can be characterized by fever, rigors, sweating, pallor, and other manifestations. It is endemic in many parts of Indonesia including Mandailing Natal Regency in North Sumatera. Current recommendation treatment for malaria is artemisinin combination therapy with primaquine. However, there is little evidence of the efficacy of the drugs in Indonesia. Therefore, this study is performed to assess the efficacy of current treatment particularly for *Plasmodium falciparum* malaria in North Sumatera and to obtain additional information whether there is resistance to antimalarial among infected participants.

2. Why have I been chosen?

Malaria can affect you or your child, the symptoms can disturb daily activities and in children can influence attendance in school. If you or your children suffered from falciparum malaria, we will ask you to participate in the study to help us in determining the malaria situation in your place and the best treatment option for you in the future.

3. Do I have to take part?

It is up to you to decide to join the study. We will describe the study and go through this information sheet. If you agree to take part, we will then ask you to sign a consent form. You are free to withdraw at any time, without giving a reason. This will not affect the standard of care you receive.

4. What will happen to me if I take part?

- A small amount of blood will be taken to screen malaria infection by microscopy examination, rapid diagnostic test and also for PCR sampling.
- If you are eligible to be one of the participants, then we will draw another small amount of blood on the following day to check your hemoglobin.
- You will be randomised and treated for malaria infection with either dihydroartemisinin-piperaquine or artemether-lumefantrine, then you will be followed for about 1.5 months (up to day 42) to see the result of treatment. Therefore, on scheduled days 1, 2, 3, 7, 14, 21, 28, 35 and 42 we will asked you to come to the study site to repeat the procedure again.
- If in any day you (or your child) feel illness, we ask you to contact us so we can visit you to do further assessment.
- If in any case you (or your child) miss an appointment, you will be visited by one of our staff in your home to find the reason of your delay.
- If in case the treatment given to you (or the child) is not working well, it will be changed with alternative drug according to the national treatment protocol.

5. Expenses and payments

The expenses for your (or your child) check-up, required tests and treatment during the study will be free of charge. Public transport (exceptionally taxi fare) for coming to and from study site will be reimbursed on every scheduled visit. There will be no any other payment.

6. What do I have to do?

We expect you to attend every scheduled days for follow up on physical examination and blood sampling.

7. What are the alternatives for diagnosis or treatment?

The gold standard for malaria diagnosis is by microscopy, therefore we will perform blood sampling to prepare the slide for microscopy examination. And later we will confirm the diagnosis by PCR.
The given treatment is currently the national guideline for treatment of falciparum malaria, if you do not respond well after treatment we will give you the alternative drug according to national protocol.

8. What are the possible disadvantages and risks of taking part?

- Some common side effects including nausea, headache, dizziness which are common for most drugs may occur, which are mild and tolerable.
- In some cases, severe malaria might still develop even after administration of study treatment. In this case, you will be treated with standard therapy for severe malaria.
- You will be asked to do blood sampling in each visit, and will be closely monitoring during the course of the study. Blood sampling may cause minimum discomfort, but will be performed by experienced staff.

9. What are the possible benefits of taking part?

We cannot promise the study will help you but the information we get might help in choosing the best treatment for falciparum malaria in the future.

10. What happens when the research study stops?

On completing of the study, no further participation will be required.

11. Will my taking part in the study be kept confidential?

Yes. All information collected about you during the course of the research will be kept strictly confidential.

12. What if relevant new information becomes available?

Sometimes during the course of a research project, new information becomes available about the treatment that is being studied. If this happens, your research doctor will tell you about it and discuss whether you want to or should continue in the study. If you decide not to carry on, your research doctor will make arrangements for your care to continue. If you decide to continue in the study you will be asked to sign an updated consent form.

If the study is stopped for any other reason, you will be told why and your continuing care will be arranged.

13. What will happen if I don't want to carry on with the study?

You can withdraw from treatment but keep in contact with us to let us know your progress. Information collected may still be used. Any stored blood samples that can still be identified as yours will be destroyed if you wish.

14. What if something goes wrong?
If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions.

If you are harmed due to someone's negligence, then you may have grounds for a legal action. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been treated during the course of this study then you should immediately inform the Investigator Dr. Inke Lubis +628126022248.

15. What will happen to the results of the research study?
The result of the study will be reported to the Indonesian government as contribution to set up malaria treatment guideline in the future, and if significant will also be published. None of the participant will be identified in any report/publication.

16. Who is organising and funding the research?
University of Sumatera Utara, Indonesia and London School of Hygiene & Tropical Medicine, United Kingdom.

17. Who has reviewed the study?
This study was given a favourable ethical opinion by the University of Sumatera Utara Research Ethics Committee and London School of Hygiene & Tropical Medicine Research Ethics Committee.

18. Contact Details

Dr. Inke Lubis +628126022248

For studies using human tissue:

19. What will happen to any samples I give?
The purified DNA extracts and unused filter paper samples will be kept at London School of Hygiene & Tropical Medicine. Final data sets will be sent to Indonesian collaborators at the conclusion of our analysis.

20. Will any genetic tests be done?
No.

You will be given a copy of the information sheet and a signed consent form to keep.

Thank you for considering taking the time to read this sheet.

Appendix 12. Informed consent form

INFORMED CONSENT FORM

Full Title of Project: **CLINICAL EFFICACY OF ARTEMISININ-BASED COMBINATION THERAPY FOR TREATMENT OF UNCOMPLICATED *PLASMODIUM FALCIPARUM* MALARIA IN NORTH SUMATERA, INDONESIA AND THE ASSOCIATION OF MOLECULAR MARKERS WITH TREATMENT OUTCOMES**

Name of Principal Investigator: Inke Lubis

	Please initial box
1. I confirm that I have read and understand the participant information sheet dated ..... (version 1) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered fully.	
2. I understand that my participation is voluntary and I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	
3. I understand that sections of my medical notes and data collected during the study may be looked at by responsible individuals from the London School of Hygiene & Tropical Medicine, from regulatory authorities or from this hospital, where it is relevant to my taking part in this research. I give permission for these individuals to access my records.	
4. I agree to take part in the above study.	
5. I agree for any remaining tissue samples to be retained, stored at LSHTM and University of Sumatera Utara and used for future research, subject to further ethical approval	

Name of Participant *(printed)*	Signature/Thumbprint	Date
Name of Person taking consent	Signature	Date
Principal Investigator	Signature	Date

The participant is unable to sign. As a witness, I confirm that all the information about the study was given and the participant consented to taking part.

Name of Impartial Witness *(if required)*	Signature	Date

1 copy for participant; 1 copy for Principal Investigator; 1 copy to be kept with hospital notes

Page 1 of 1 Version 1.0; <30/06/2014>

Appendix 13. Participant information sheet in Bahasa Indonesia

Lembar Penjelasan Pasien

Judul Penelitian:
UJI EFIKASI KLINIS DARI TERAPI KOMBINASI ARTEMISININ UNTUK TATALAKSANA INFEKSI MALARIA *PLASMODIUM FALCIPARUM* TANPA KOMPLIKASI DI SUMATERA UTARA, INDONESIA, DAN HUBUNGAN ANTARA PENANDA MOLEKULAR DENGAN HASIL AKHIR TERAPI

Anda diundang untuk berpartisipasi dalam suatu penelitian. Sebelum Anda memutuskan untuk ikut dalam penelitian, penting bagi Anda untuk mengerti kenapa penelitian ini dilakukan dan apa saja yang akan dilakukan. Mohon berikan waktu Anda untuk membaca informasi berikut dan berdiskusi dengan lainnya mengenai penelitian ini jika perlu. Anda dapat bertanya kepada kami jika ada bagian yang tidak jelas atau jika Anda menginginkan informasi lebih lanjut. Ambil waktu yang Anda butuhkan sebelum mengambil keputusan untuk ikut ataupun tidak ikut serta dalam penelitian ini.

1. Apakah tujuan dari penelitian ini?

Malaria merupakan penyakit infeksi yang disebabkan oleh parasit Plasmodium yang ditularkan oleh nyamuk. Gejala malaria ditandai dengan demam, menggigil, berkeringat, pucat dan lainnya. Malaria merupakan endemik di berbagai daerah di Indonesia termasuk Kabupaten Mandailing Natal, Sumatera Utara. Rekomendasi terapi pada saat ini ialah dengan kombinasi terapi artemisinin (ACT) dengan primakuin. Namun, masih sedikit penelitian mengenai efikasi pengobatan ini di Indonesia. Sehingga, penelitian ini dilakukan untuk menilai efikasi dari standard terapi pada saat ini untuk infeksi Plasmodium falciparum di Sumatera Utara, dan untuk mendapatkan informasi lebih lanjut apakah terdapat resistensi terhadap antimalarial.

2. Mengapa saya dipilih?

Malaria dapat mempengaruhi Anda ataupun anak Anda, gejala malaria dapat mengganggu aktivitas sehari-hari dan pada anak dapat mengganggu kehadiran di sekolah. Jika Anda atau anak Anda menderita malaria falsiparum, kami akan meminta Anda untuk berpartisipasi dalam studi ini untuk membantu kami dalam menentukan situasi malaria di daerah Anda dan pilihan terapi yang terbaik bagi Anda di masa depan.

3. Apakah saya harus berpartisipasi?

Keputusan berada di tangan Anda. Kami akan memberikan penjelasan mengenai penelitian ini. Jika Anda setuju untuk berpartisipasi, maka kami akan meminta anda untuk menandatangani surat persetujuan. Anda dapat mengundurkan diri kapan saja, tanpa harus memberikan penjelasan. Hal ini tidak akan mempengaruhi terapi standard yang Anda terima.

4. Apa yang terjadi jika saya berpartisipasi?

- Sedikit darah akan diambil dan diperiksa untuk infeksi malaria dengan pemeriksaan mikroskopis, uji diagnostik cepat dan juga untuk pemeriksaan PCR.
- Jika Anda memenuhi syarat untuk menjadi peserta,maka sejumlah kecil darah akan diambil pada hari berikutnya untuk memeriksa kadar hemoglobin Anda
- Anda akan diacak dan menerima terapi untuk infeksi malaria dengan kombinasi dihidroartemisinin-piperakuin atau dengan kombinasi artemether-lumefantrin, lalu Anda akan diawasi selama 1,5 bulan (42 hari) untuk menilai respon terapi. Anda akan diminta untuk datang ke klinik pada hari 1, 2, 3, 7, 14, 21, 28, 35 dan 42 untuk mengulangi pemeriksaan.

- Jika pada hari apapun Anda (atau anak Anda) merasa sakit, kami meminta Anda untuk menghubungi staf kami agar kami dapat mengunjungi Anda untuk melakukan pemeriksaan selanjutnya.
- Jika Anda (atau anak Anda) melewati pada satu kunjungan, Anda akan dikunjungi oleh salah satu staf kami di rumah Anda untuk mengetahui alasan ketidakhadiran Anda.
- Jika pengobatan yang diberikan kepada Anda (atau anak Anda) tidak memberikan respon yang sesuai, maka terapi akan diganti dengan terapi alternatif sesuai rekomendasi nasional.

5. Biaya

Biaya untuk pemeriksaan dan terapi Anda (ataupun anak Anda) selama studi ditanggung oleh penelitian ini. Transportasi umum (kecuali taksi) untuk datang dan pulang dari klinik tempat penelitian akan diganti untuk setiap kunjungan. Tidak ada biaya lainnya yang diberikan.

6. Apakah yang harus saya lakukan?

Kami mengharapkan Anda untuk hadir pada setiap hari kunjungan yang telah ditetapkan untuk pemeriksaan fisik dan pengambilan darah.

7. Apakah pilihan lain dari diagnosis dan terapi?

Baku standard untuk diagnosis malaria ialah dengan mikroskopi, sehingga kami akan mengambil darah Anda untuk pemeriksaan mikroskopis. Kemudian kami juga akan melakukan pemeriksaan dengan PCR.
Terapi yang diberikan merupakan acuan nasional untuk terapi malaria falsiparum, jika Anda tidak memberikan respon yang sesuai setelah terapi maka kami akan mengganti dengan terapi lain sesuai acuan nasional.

8. Apakah kerugian ataupun risiko dari ikut berpartisipasi dalam penelitian?

- Beberapa efek samping seperti mual, pusing, sakit kepala dapat terjadi, yang merupakan efek samping dari kebanyakan obat, tetapi umumnya ringan dan dapat ditoleransi
- Pada kasus tertentu, malaria berat masih dapat terjadi setelah pemberian terapi. Dalam kasus ini, Anda akan diterapi dengan terapi standard untuk malaria berat
- Darah Anda akan diambil pada setiap kunjungan, dan Anda akan diawasi selama studi berlangsung. Pengambilan darah dapat menyebabkan ketidaknyamanan, tetapi hal ini akan dilakukan oleh staf yang sudah berpengalaman.

9. Apakah keuntungan dari berpartisipasi?

Kami tidak dapat menjamin bahwa penelitian ini dapat membantu Anda tetapi informasi yang kami terima akan membantu dalam menentukan terapi terbaik untuk infeksi malaria falsiparum di masa yang akan datang.

10. Apa yang akan terjadi ketika penelitian telah selesai?

Ketika penelitian selesai dilaksanakan, partisipasi lebih lanjut tidak dibutuhkan lagi.

11. Apakah kerahasiaan saya akan dijaga?

Ya. Seluruh informasi mengenai Anda selama penelitian akan dijaga kerahasiaannya.

12. Apa yang akan terjadi jika terdapat informasi baru?

Terkadang dalam penelitian, informasi baru mengenai terapi dapat diterima. Jika ini terjadi, dokter Anda akan memberitahu Anda dan berdiskusi dengan Anda jika Anda masih ingin melanjutkan penelitian atau tidak. Jika Anda memutuskan untuk tidak melanjutkan penelitian, dokter Anda akan mengatur agar terapi Anda tetap dilanjutkan. Jika Anda memutuskan untuk tetap berpartisipasi, Anda akan diminta untuk menandatangani surat persetujuan yang terbaru.

Jika penelitian dihentikan oleh karena alasan tertentu, Anda akan mendapatkan penjelasan dan terapi tetap akan dilanjutkan.

13. Apa yang akan terjadi jika saya tidak ingin lagi terlibat dalam penelitian?
Anda dapat mengundurkan diri dari terapi tetapi tetap berhubungan dengan kami untuk mengetahui kelanjutan Anda. Informasi yang telah dikumpulkan tetap akan digunakan. Sampel darah yang sudah diambil dan dapat diidentifikasi sebagai milik Anda akan dimusnahkan jika Anda inginkan.

14. Apa yang akan terjadi jika terdapat sesuatu yang salah?
Jika anda mempunyai kekhawatiran terhadap aspek apapun dari penelitian ini, Anda dapat membicarakannya dengan peneliti yang akan membantu memberikan penjelasan kepada Anda.

Jika Anda mengalami cedera karena kelalaian seseorang, maka Anda dapat melakukan tindakan legal. Diluar keadaan ini, Anda tetap dapat memberikan keluhan, atau jika Anda mempuyai kekhawatiran mengenai bagaimana Anda diperlakukan selama penelitian, Anda dapat segera menghubungi peneliti utama Dr. Inke Lubis +628126022248.

15. Apa yang akan terjadi dari hasil penelitian ini?
Hasil penelitian ini akan dilaporkan ke pemerintah Indonesia sebagai kontribusi dalam menentukan acuan tatalaksana malaria di masa yang akan datang. Hasil penelitian juga akan dipublikasikan. Identitas peserta penelitian tidak akan terdapat didalam laporan ataupun publikasi.

16. Siapakah yang menyelenggarakan dan mendanai penelitian ini?
Universitas Sumatera Utara, Indonesia dan London School of Hygiene & Tropical Medicine, Inggris.

17. Siapakah yang telah meninjau penelitian ini?
Penelitian ini ditinjau secara etik oleh Komite Etik Universitas Sumatera Utara dan Komite Etik London School of Hygiene & Tropical Medicine.

18. No. Kontak

Dr. Inke Lubis +628126022248

Untuk penelitian yang melibatkan jaringan manusia:

19. Apakah yang akan terjadi pada sampel yang saya berikan?
DNA dan kertas filter yang tidak digunakan akan disimpan di London School of Hygiene & Tropical Medicine. Data akhir akan dikirim ke kolaborator di Indonesia setelah analisis.

20. Apakah tes genetik akan dilakukan?
Tidak.

Anda akan mendapatkan kopi dari lembar penjelasan dan lembar persetujuan yang telah ditanda tangani untuk disimpan.

Terima kasih telah membaca lembar penjelasan ini.

Appendix 14. Informed consent form in Bahasa Indonesia

LONDON SCHOOL of HYGIENE & TROPICAL MEDICINE

LEMBAR PERSETUJUAN

Judul Penelitian: **UJI EFIKASI KLINIS DARI TERAPI KOMBINASI ARTEMISININ UNTUK TATALAKSANA INFEKSI MALARIA *PLASMODIUM FALCIPARUM* TANPA KOMPLIKASI DI SUMATERA UTARA, INDONESIA, DAN HUBUNGAN ANTARA PENANDA MOLEKULAR DENGAN HASIL AKHIR TERAPI**

Nama Peneliti Utama: Inke Nadia D. Lubis

	Mohon ditandai ✓
1. Saya telah membaca dan mengerti lembar penjelasan pasien yang bertanggal 30 Juni 2014 (versi 1) untuk penelitian diatas. Saya telah mendapat kesempatan untuk mempertimbangkan informasi yang saya terima, bertanya dan mendapatkan jawaban.	
2. Saya mengerti bahwa keikutsertaan saya ialah tanpa paksaan dan saya bebas untuk mengundurkan diri kapan saja, tanpa harus memberikan alas an, dan perawatan medis saya tidak akan terpengaruhi.	
3. Saya mengerti bahwa data medis saya yang dikumpulkan selama penelitian akan dilihat oleh individu yang bertanggungjawab di London School of Hygiene & Tropical Medicine, dari pihak berwenang ataupun dari klinik penelitian ini, selama hal tersebut berhubungan dengan penelitian ini. Saya memberi izin terhadap pihak-pihak tersebut untuk dapat mengakses rekam medis saya.	
4. Saya bersedia untuk ikut serta dalam penelitian diatas.	
5. Saya setuju agar sisa sampel yang diambil untuk disimpan di London School of Hygiene & Tropical Medicine dan Universitas Sumatera Utara dan dapat digunakan untuk penelitian di masa datang, dengan persetujuan etik yang baru.	

Nama Peserta *(cetak)*	Tanda tangan/Sidik jari	Tanggal
Nama yang Mengambil Izin	Tanda tangan	Tanggal
Peneliti Utama	Tanda tangan	Tanggal

Jika peserta tidak dapat menandatangani. Sebagai saksi, saya menjamin bahwa seluruh informasi mengenai penelitian telah diberikan dan peserta bersedia untuk berpartisipasi.

Nama Saksi *(jika perlu)*	Tanda tangan	Tanggal

1 kopi untuk peserta; 1 kopi untuk peneliti utama; 1 kopi untuk disimpan sebagai berkas klinik//rumah sakit.

Page 1 of 1 Version 1.0; <30/06/2014>

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Malaria Journal

RESEARCH Open Access

Malaria epidemiology in central Myanmar: identification of a multi-species asymptomatic reservoir of infection

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Abstract

Background: The spread of artemisinin-resistant *Plasmodium falciparum* is a global health concern. Myanmar stands at the frontier of artemisinin-resistant *P. falciparum*. Myanmar also has the highest reported malaria burden in South-east Asia; it is integral in the World Health Organization's plan to eliminate malaria in Southeast Asia, yet few epidemiological data exist for the general population in Myanmar.

Methods: This cross-sectional, probability household survey was conducted in Phyu township, Bago Region (central Myanmar), during the wet season of 2013. Interviewers collected clinical and behavioural data, recorded tympanic temperature and obtained dried blood spots for malaria PCR and serology. *Plasmodium falciparum* positive samples were tested for genetic mutations in the K13 region that may confer artemisinin resistance. Estimated type-specific malaria PCR prevalence and seroprevalence were calculated, with regression analysis to identify risk factors for seropositivity to *P. falciparum*. Data were weighted to account for unequal selection probabilities.

Results: 1638 participants were sampled (500 households). Weighted PCR prevalence was low (n = 41, 2.5%) and most cases were afebrile (93%). *Plasmodium falciparum* was the most common species (n = 19. 1.1%) and five (26%) *P. falciparum* samples harboured K13 mutations. *Plasmodium knowlesi* was detected in 1.0% (n = 16) and *Plasmodium vivax* was detected in 0.4% (n = 7). Seroprevalence was 9.4% for *P. falciparum* and 3.1% for *P. vivax*. Seroconversion to *P. falciparum* was 0.003/year in the whole population, but 16-fold higher in men over 23 years old (LR test p = 0.016).

Discussion: This is the first population-based seroprevalence study from central Myanmar. Low overall prevalence was discovered. However, these data suggest endemic transmission continues, probably associated with behavioural risk factors amongst working-age men. Genetic mutations associated with *P. falciparum* artemisinin resistance, the presence of *P. knowlesi* and discrete demographic risk groups present opportunities and challenges for malaria control. Responses targeted to working-age men, capable of detecting sub-clinical infections, and considering all species will facilitate malaria elimination in this setting.

Keywords: Malaria, Myanmar, Prevalence, Serology, Transmission, Artemisinin, Resistance, Risk factors, Elimination

Background

The last decade has seen global progress in malaria control [1, 2]. This is threatened by the emergence of *Plasmodium falciparum* resistant to artemisinin, the world's front-line anti-malarial [3]. So far, *P. falciparum* with reduced susceptibility to artemisinin has been described in Cambodia, Thailand, Vietnam, Laos and Myanmar [4–7]. The World Health Organization (WHO) recently set its sights on malaria elimination in Southeast Asia in order to contain this threat [2]. The emergence of the zoonotic primate malaria *Plasmodium knowlesi* as a

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parasite of significant public health importance in Malaysian Borneo complicates elimination [8, 9]; the distribution and prevalence of this species in other countries in the region remains uncertain.

Myanmar is reported to account for the vast majority of malaria cases and deaths within Southeast Asia [10]. Better understanding the epidemiology of malaria here would likely contribute to successful elimination. Malaria distribution in Myanmar is heterogeneous; forests are a major environmental factor driving patterns of disease [11, 12] and international and internal migration are also risk factors [13, 14]. Whilst transmission is relatively well documented in border areas [13], few data exist from central Myanmar and the burden and drivers of infection in this area remain poorly understood.

Light microscopy and rapid diagnostic tests (RDTs) underestimate the prevalence of infection with *Plasmodium* spp, particularly in low-endemic settings [15]. Polymerase chain reaction (PCR) is more sensitive in detecting current infection and allows further testing such as for drug resistance-associated mutations [16]. Serological methods have been shown to reliably estimate malaria transmission and can identify risk factors with achievable sample sizes in low prevalence areas [17]. Prevalence of antibodies to specific malaria antigens, which indicate malaria exposure, can be used to calculate the seroconversion rate (SCR, equivalent to the force of infection) [18]. Changes in SCR over time are of particular utility in monitoring the success of public health campaigns.

This study combined highly sensitive PCR testing and serological evidence of transmission intensity in a household survey in central Myanmar with collection of detailed epidemiological data to investigate the prevalence of, and risk factors for, malaria exposure.

Methods

Study site and population

Phyu is the largest township in Eastern Bago Region. Phyu is located on the main highway between Yangon (largest city) and Nay Pyi Taw (capital). It is 100 km west of the border with Thailand, and lies on a plain (highest altitude = 60 m) between forest-covered mountains (Fig. 1). The forests attract migrant labour for logging, charcoal production and agro-forestry [14]. Phyu was selected for this study due to its relatively large size and location on busy transport routes near forests. The population is approximately 250,000, of whom 89% live in semi-urban or rural areas, with 11% in Phyu town.

Medical services vary from a 50-bed township hospital to sub-rural health centers with no inpatient facilities. The latter cover 5–15 villages each with a midwife who provides basic services including malaria prevention. The township malaria inspector and local midwives accompanied the team for this study.

The monsoon begins in May and ends in October; precipitation is heaviest in August (approximately 450 mm/month). Peaks in malaria transmission follow the rains by 1–2 months. This study was conducted in August and September 2013.

Field procedures

Sample size calculation

A required sample size of 1174 was calculated, assuming a malaria prevalence of 1 per 1000, with 95% confidence with 80% power; sampling 0.1% of the population, with a presumed design effect of 2 and a non-response rate of 15%. Since the average household size in Myanmar is 4.7, 30 clusters of 20 houses (~75 participants) were chosen (n = 1500).

Sampling technique

This cross-sectional study used a multistage cluster random sample design. Administrative wards were the primary sampling unit, with villages and houses as the secondary and tertiary sampling units. 26 of 61 wards were included (population = 152,651, 60.2% of the total). The remaining wards (population = 101,078, 39.8%) were defined as inaccessible by vehicle by the local government, necessitating exclusion. The included wards were weighted according to population size and randomly selected.

From each selected ward, villages were selected at random with no population weighting. If a ward was selected more than once, more than one village was selected. Each selected village represented one cluster. A map was drawn by village leaders and houses were selected by systematic interval sampling and mapped using a Global Positioning System. Houses with no response were not revisited, the house with the closest street entrance was sampled in its place.

Questionnaires

Questionnaires were adapted from publicly available malaria indicator survey toolkits [19]. Pre-tested questionnaires were administered by pairs of community healthcare workers trained for this study. Households were defined as the group of people who had slept under the same roof the previous night and who shared a common entrance to the street. Household questionnaires recorded household structure, assets and demographic information for every household member, including those absent. Individual questionnaires recorded self-reported forest exposure, malaria symptoms and malaria prevention practices.

Participants were categorized as having exposure to forest if their occupation involved forest-related work

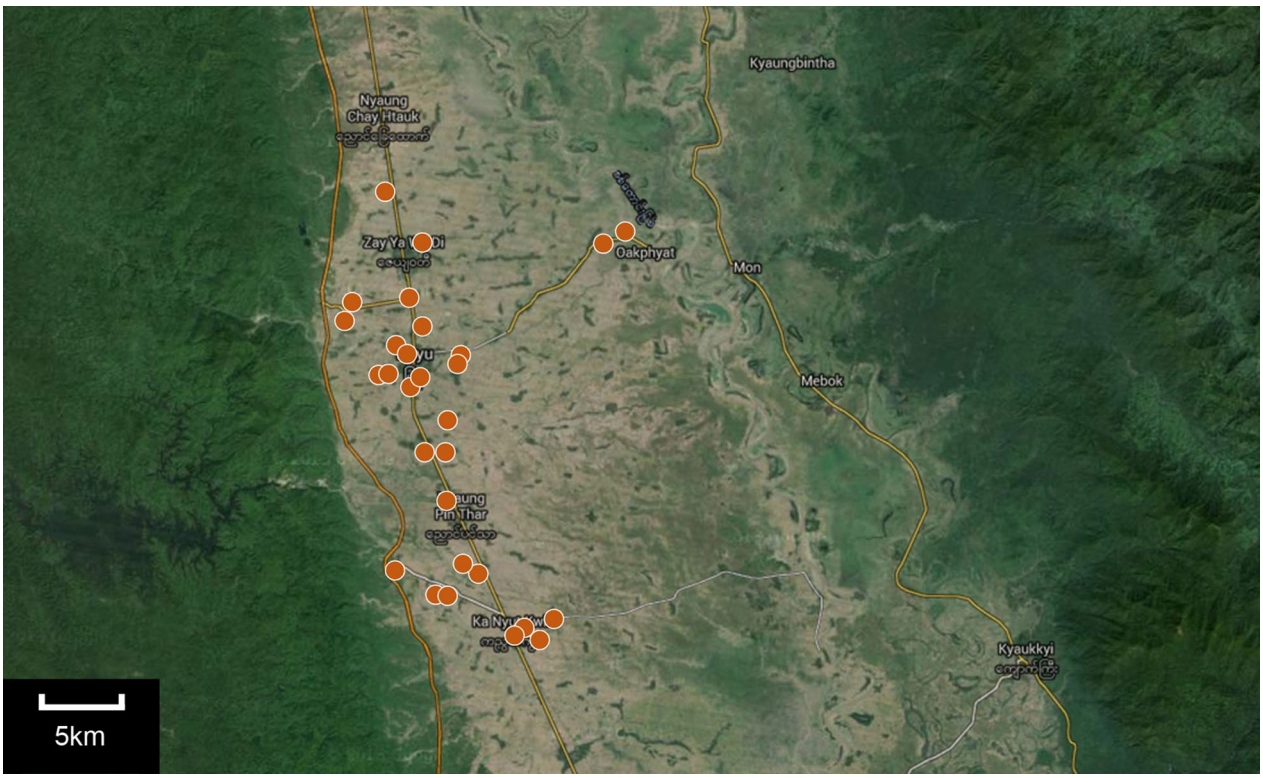

Fig. 1 Study site with GPS points (*circles*) demarcating each village cluster

(forestry, farming, labouring) or if they reported visiting the forest within the previous 6 months. Households were classified as having universal coverage of bed nets if they reported having at least 1 net per 2 people. Socioeconomic status (SES) was calculated using principal components analysis including household structure and assets. The derived score was divided into tertiles.

Clinical samples

Tympanic temperature was measured for all participants. Febrile participants (≥37.5 °C) were tested using RDT (SD Bioline Malaria Ag Pf/Pan) and referred for treatment. Capillary blood samples were spotted directly onto Whatman 3MM filter paper for all participants over 6 months old regardless of tympanic temperature. Blood spots were air-dried, sealed with silica gel and sent to the London School for Hygiene and Tropical Medicine for storage at −20 °C before analysis.

Ethical approval

The study received approval from the Research Ethics Committee of University College London, the Myanmar Ministry of Health and Department of Medical Research, Lower Myanmar. Local and regional authorities were informed. Informed consent was obtained from all adults and from guardians of children aged under 15. For those aged between 15 and 18, informed consent from both parent and child was obtained.

Laboratory procedures

Serological analysis

Recombinant proteins: *Plasmodium falciparum* and *Plasmodium vivax* antigens MSP-1_{19} were expressed in *Escherichia coli* as glutathione *S*-transferase-fusion proteins [20] (Pv provided by A. Holder). PfAMA1_3D7 and PvAMA1_Sal1 were expressed as His-tagged proteins [21, 22].

Detection of anti-malarial antibodies by ELISA: IgG antibodies to each recombinant protein were assessed by ELISA as previously described [23]. Briefly, recombinant antigens were coated overnight at 4 °C at the following concentrations: PfAMA-1 at 0.5 μg/ml, PfMSP-1_{19} at 0.18 μg/ml, PvAMA-1 at 1 μg/ml and PvMSP-1_{19} at 1.8 μg/ml. Plates were washed using PBS plus 0.05% Tween 20 (PBS/T) and blocked with 1% skimmed milk powder in PBS/T. Samples and positive control sera (a pool of hyperimmune serum collected from a malaria endemic area) were tested in duplicate at 1/1000 and 1/2000 dilutions against MSP and AMA, respectively. After washing, horseradish peroxidase-conjugated

polyclonal rabbit anti-human IgG (Dako, Glostrup, Denmark) diluted at 1:5000 in PBS/T was added to all wells. All plates were developed using OPD substrate (OPD, sigma fast) and reactions were stopped with 2 M H_2SO_4. Plates were read at 492 nm and optical density (OD) values were recorded. Seropositivity was determined using a finite mixture model to define a cut-off value as previously described [18].

PCR analysis

Parasite DNA from blood spots was extracted using the Chelex method [24]. PCR amplifications targeting the 18S rRNA gene was performed for initial screening for *Plasmodium* infections, using published PCR reactions and protocols with minor modifications [8]. PCR-positive samples were subsequently tested using an additional method targeting the cytochrome B gene (*cytb*) designed for the current study. The primer sets for the hemi-nested *cytb* assay were as follows: PgCytbF1 (5′-GAATTATG GAGTGGATGGTG-3′) and PgCytbR1 (5′-ACATC CAATCCATAATAAAGC-3′) for nest 1 and PgCytbF1 and PgCytbR2 (5′-TTTTAACATTGCATAAAATGG-3′) for nest 2. Amplification cycling conditions were 95 °C for 3 min and then 30 cycles of 3 steps PCR, with 30 s annealing at 55 °C (nest 1) or 58 °C (nest 2) and elongation at 72 °C for 1 min. Nested products were then characterized by direct sequencing for species determination. Polymorphisms at the propeller domain of the Kelch 13 (K13) encoded by the *P. falciparum k13* gene (PF3D7_1343700) were also determined on *P. falciparum* PCR-positive isolates, using previously described protocols for nested PCR methods and DNA sequencing [25].

Microscopic analysis

Thick and thin blood films were taken from each participant. Giemsa-stained thick malaria films were examined under a 100× oil-immersion lens. Samples were counted as negative if no parasite was seen after counting 400 white blood cells. Samples were counted as positive if one or more parasites were seen—the corresponding thin films were then examined with a 100× oil-immersion lens for speciation. All microscope slides were read in duplicate by technicians blinded to other reader's findings.

Data analysis

Weighted prevalence of malaria species by PCR and serological status was estimated with 95% confidence intervals using the Stata survey commands (v14, Statacorp, Texas).

To investigate transmission dynamics in the population, a simple reversible catalytic conversion model was fitted to the age-seroprevalence data using maximum likelihood methods as previously described [17]. The model fits age-seroprevalence curves and estimates seroconversion rate (SCR) as analogous to the force of infection. Profile likelihood plots were used to test for any step change in SCR and, if present, identify the most likely age at which this occurred [26]. Models with two SCRs instead of one were assumed if the likelihood ratio comparing models indicated a statistically significant change ($p < 0.05$).

Univariate and multivariate logistic regression was used to investigate the relationship between gender, age, ethnicity, bed net ownership, SES, forest exposure, and seropositivity to *P. falciparum* antigens.

Results

A total of 701 houses in 27 clusters (villages) across 14 administrative wards were visited, of which 500 houses had at least one resident present. Of the 2112 recorded residents of consenting households, 474 (22.4%) were absent (Table 1). 1638 were present and all consented to take part in the study.

The median age was 30 years (range 6 months-88 years) and 55% were female. 43% (57% of men and 27% of women) were classified as having had forest exposure (Table 1). 80% of households were classified as having universal bed net coverage, but very few households were reported as having been sprayed with insecticide in the past year (3%). The majority of participants were of Burmese origin (63%) whilst people of Indian origin represented 33% of the sample and Shan origin 3%. Absent household residents were more likely to be male and less likely to be Indian compared to those included in the study.

Malaria indicators

41 participants were found to have current infection when tested post hoc by PCR (2.6%, CI 1.5–4.3) (Table 2). The most common species detected was *P. falciparum* (1.1%, n = 19), followed by *P. knowlesi* (1.0%, n = 16). *Plasmodium vivax* (0.5%, n = 7) and *Plasmodium malariae* (0.08%, n = 1) (Fig. 2). Only two mixed infections were detected. Three infected individuals were febrile (one each of *P. falciparum, P. knowlesi* and *P. vivax*), the remaining 38 were afebrile (93%). No infected individuals reported any other malaria symptoms and none had sought treatment for malaria in the past three months. 89 febrile people were tested with an RDT and none were found positive, including three PCR-confirmed infections. *P. falciparum* and *P. knowlesi* infections were found in all age groups. *Plasmodium knowlesi* was most common between ages 5 and 15. *Plasmodium vivax* was only detected in those aged 15 and above.

5 of the 19 (26.3%) *P. falciparum* samples harboured non-synonymous propeller domain mutations in

Table 1 Demographic characteristics of study participants

	Study population	
	n	%
Total	1638	100
Gender		
Males	752	45.9
Female	886	54.1
Age		
0–5	163	9.9
6–15	324	19.8
16–25	255	15.6
26–50	550	33.6
51–100	346	21.1
Ethnicity		
Burmese	1086	64.1
Indian	507	33.0
Shan	45	2.9
Socioeconomic status		
Lowest	513	32.4
Middle	549	34.6
Highest	523	33.0
Work in or visit forest		
Yes	671	41.0
No	967	59.0
Household bed net ownership		
Yes, universal coverage	398	79.6
No	102	20.4

pfk13 (K13). These included the known variant Val534Ala [27] and four novel variants: Arg539Val, Asn572Asp, Glu602Gly, and Glu620Gly.

9.4% (n = 150) individuals were seropositive to *P. falciparum* antigens whilst 3.1% (n = 50) were seropositive to *P. vivax* antigens. Of the 50, 19 (38%) were also positive to *P. falciparum*, indicating exposure to both species. Seropositivity to both *P. falciparum* and *P. vivax* was higher in men than women for both species (p value = 0.018 and 0.063 respectively).

Microscope slide positivity had high inter-observer variability, in part due to water damage and fungal contamination of slides, and the microscopy results were therefore not included in the analysis.

Risk factors for exposure to malaria

Analysis of risk factors for malaria was restricted to seropositivity to *P. falciparum* due to the low numbers of infections detected by PCR. After adjusting for all other variables, seropositivity was more likely for men, [adjusted OR (AOR) 2.1 (1.3–3.4), p = 0.006], and less likely for those with higher SES [AOR 0.6 (0.3–0.9), p = 0.072], and of Indian origin [AOR 0.3 (0.1–0.8)]. There was a strong association between seropositivity and forest exposure in the unadjusted model, which was lost after adjusting for gender, age, ethnicity and SES (Table 3). Migration status (moving to Phyu within the last 10 years or visiting outside Phyu in the preceding 3 months) was not associated with an increased risk of malaria exposure in either the crude or adjusted analysis.

Serological estimates of malaria transmission

A reversible catalytic model and profile likelihood plots were used to investigate whether there was evidence for two SCRs, indicative of two discrete levels of exposure within the population. For *P. falciparum*, there was a clear peak in profile likelihood plot at age 23 years, indicating a change in SCR (Fig. 3a). A model allowing for two SCRs fitted the data significantly better than a model with a single SCR (p = 0.016) (Fig. 3b).

When analysed separately by gender, a model with two SCRs fitted better for men (p = 0.004) whilst a single SCR fit better for women (p = 0.548) (Fig. 4). The SCR in men over the age of 23 was 0.050 (0.010–0.251)

Table 2 Prevalence of symptoms (fever) and specific *Plasmodium* species by PCR and serology

	n	Prevalence (95% CIs)	Proportion of current infection (%)
Fever	89	4.9 (3.5–7.1)	3/89 (3.4%)
PCR			
Any *Plasmodium* species	41	2.6 (1.5–4.3)	100[a]
P. falciparum	19	(0.4–3.0)	46.3 (28.7–59.1)
P. knowlesi	16	(0.5–2.2)	39.0 (21.9–51.3)
P. vivax	7	0.5 (0.1–2.0)	17.1 (2.2–22.2)
P. malariae	1	0.04 (0.0–0.4)	2.4 (0–7.08)
Seropositive			
P. falciparum	150	9.4 (6.0–15.0)	
P. vivax	50	3.1 (1.9–6.3)	

[a] Includes two mixed infections, one *P. falciparum* and *P.vivax,* one *P. knowlesi* and *P. vivax*, therefore total percentages may not equal 100%

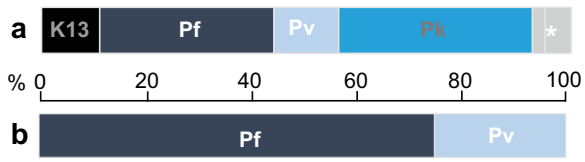

Fig. 2 Proportion of *Plasmodium* species identified in **a** our sample and **b** WHO reporting for Myanmar. Pf, *P. falciparum*; Pv, *P. vivax*; Pk, *P. knowlesi*. K13 Pf samples with K13 mutations (not available for WHO statistics). * 1 × Pf Pv, 1 × Pk Pv, 1 × *P. malariae*

compared to 0.003 (0.001–0.006) in those under 23 years old. In women, the SCR was similar to that of men under 23 [0.003 (0.001–0.005)].

Seropositivity to *P. vivax* antigens was low and SCR was also low [0.002 (0.0009–0.006)], with no evidence for a change in SCR.

Discussion

This household study reveals low-level malaria transmission among the general population in central Myanmar. The WHO estimates that *P. falciparum* causes 75% of clinical malaria in Myanmar and *P. vivax* 25% [28], whilst in this study *P. falciparum* mono-infection comprised 45% of infections and *P. vivax* 12% (Fig. 2). This study found *P. knowlesi* [29] in more than 35% of positive samples. Clinical diagnosis of malaria and most epidemiological studies in Myanmar use microscopy or RDT positivity to identify infection and both RDT and microscopy have poor sensitivity and specificity for *P. knowlesi* [30, 31]. In this study, reliable microscopy also proved technically challenging. This may explain the underrepresentation of *P. knowlesi* in national statistics and population-level data when compared to the findings presented here. In Malaysia, *P. knowlesi* is responsible for a similar proportion of cases as found in this study and can cause fatal illness [32]. The epidemiology of *P. knowlesi* in Myanmar requires further study, utilizing sensitive diagnostic tools.

One quarter of *P. falciparum* samples harboured K13 mutations, all from afebrile, asymptomatic participants, none of whom reported anti-malarial treatment within the previous three months and none were followed up—it is therefore difficult to comment on the importance of these alleles as determinants of artemisinin susceptibility. Of the five K13 mutations detected, four have never previously been reported. One of the novel mutations, Arg539Val, is similar to previously described variants at codon 539 from Cambodia, Laos and Myanmar, though

Table 3 Association between demographic and behavioural characteristics and exposure to *P. falciparum* in study participants

	Sero-positive (%)	Odds ratio (OR)	p value	Adjusted OR[a]	p value	Denominators	
						Un-weighted	Weighted
Gender							
Female	7.1	1.0 (baseline)		1.0		862	871
Male	12.6	1.9 (1.3–2.8)	0.004	2.1 (1.3–3.4)	0.006	740	731
Age							
0–5 years	0.3	1.0		1.0		162	181
5–15 years	3.1	9.0 (1.4–58.9)		9.0 (1.3–64.8)		311	292
15–25 years	3.8	11.2 (1.6–81.5)		12.0 (1.5–94.8)		253	265
25–50 years	13.0	42.7 (4.0–456.3)		43.8 (3.8–506.3)		541	544
50–100 years	20.0	71.2 (6.4–796.3)	0.014	71.3 (5.6–904.2)	0.030	335	319
Working in/visiting forests							
No	5.8	1.0		1.0		1454	1454
Yes	14.7	2.8 (1.5–5.1)	0.002	1.0 (0.6–1.8)	0.979	31	31
Bed net ownership							
Not universal coverage	6.6	1.0		1.0		391	417
Universal coverage	11.0	1.8 (0.8–3.8)	0.134	1.1 (0.5–2.5)	0.760	1161	1145
Socio-economic status							
Low	11.6	1.0		1.0		506	462
Medium	11.2	0.9 (0.6–1.5)		0.9 (0.6–1.6)		532	531
Highest	7.2	0.6 (0.4–0.9)	0.035	0.6 (0.3–0.9)	0.072	512	566

[a] Adjusted for: age, gender, forest exposure, ethnicity, bed net ownership and socio-economic status

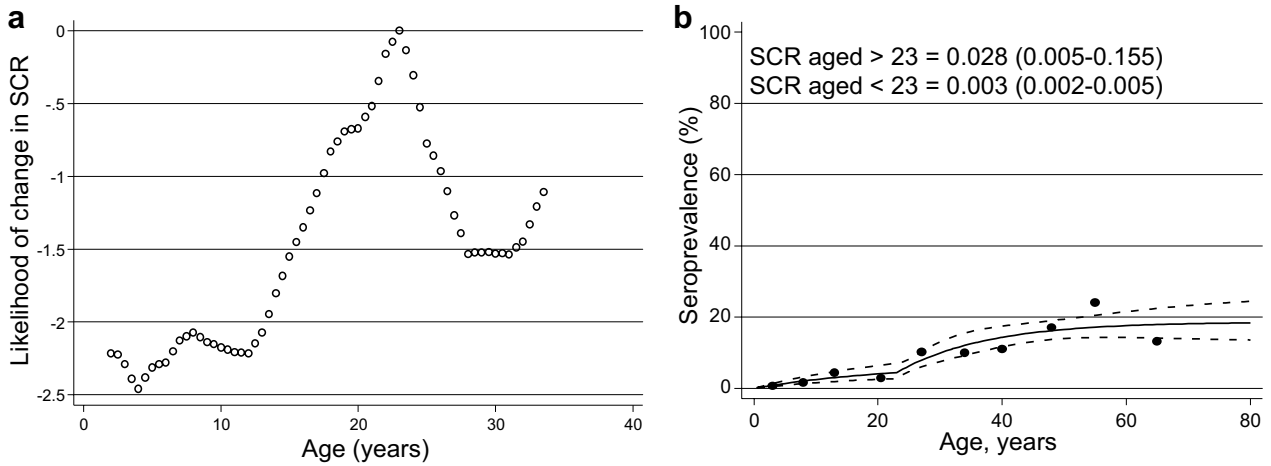

Fig. 3 **a** Profile likelihood plot investigating the most likely age at which a change in seroconversion rate (SCR) occurs in *P. falciparum* serological data and **b** Seroprevalence curve fitted to *P. falciparum* with a change in SCR at age 23

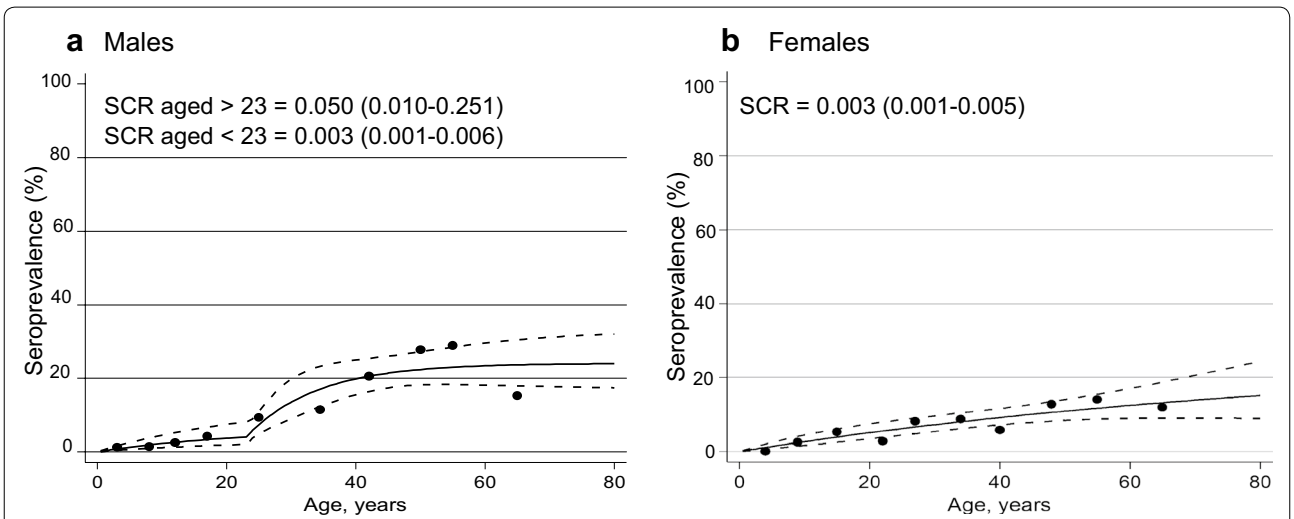

Fig. 4 Seroprevalence curves for *P. falciparum* **a** males and **b** females. The data for males has been fitted with two serconversion rates (SCR), with a change at age 23, and the data for females has been fitted with a constant SCR

these all encode threonine rather than valine as the variant amino acid [27]. K13 mutations have recently been mapped in convenience samples in Myanmar and appear prevalent in Upper Myanmar and eastern border areas; no K13 mutations were found in a clinical cohort of 52 patients in Bago Region in 2013–2014 [33], although the prevalence of K13 mutations amongst clinical cohorts in the high-risk township of Shwe Kyin, Bago, was similar to that found in our population [5].

Most participants (n = 38; 93%) with current infection were afebrile and none reported symptoms. The three individuals with raised temperatures tested negative on RDT, possibly signifying a low-density parasitaemia (or alternative diagnoses) which may be associated with mild clinical symptoms [34]. However, even sub-clinical infections are important during elimination campaigns [35]; accurate identification and treatment of asymptomatic carriers—who might transmit infections and will tend not to seek treatment—proved crucial to eliminate malaria in a similar low-endemic setting [36].

The serological data support the PCR data and suggest that there has been ongoing low-level transmission of *P. falciparum* and *P. vivax* in the general population in Bago

region. There was a notable increase in exposure to infection in men aged over 23, who experience a SCR 16-fold higher than the general population. Because the antibodies detected by these methods persist for an unknown period of time, there are two explanations indistinguishable by serology alone: malaria epidemiology may have changed 23 years ago such that men now aged over 23 were historically exposed to more malaria, or there may be ongoing behavioural risk factors specific to those aged over 23. The best working hypothesis is that the change in SCR is behavioural; it is confined to a well-defined epidemiological group (men of working age) and this population is overrepresented in contemporary clinic-based studies suggesting they remain at increased risk of malaria [16].

Forest work is an established risk factor for malaria transmission [37]. *Plasmodium knowlesi* is particularly associated with forest malaria, due to the outdoor biting habits of *P. knowlesi* vectors and the natural primate reservoirs living in forests [38]. The questionnaire captured self-reported information relating to occupational exposure and forest visits. Individuals reporting forest exposure were three times more likely to have evidence of previous exposure to malaria in the unadjusted analysis. However, forest workers were significantly more likely to be men and aged over 23 and less likely to be Indian. After adjusting for other factors including age, ethnicity and SES the association with forest exposure was no longer significant, which is consistent with forest exposure being on the causal pathway (although it cannot be excluded that it is a confounder). Illicit forest-related activities such as illegal logging are often unreliably reported in surveys, and qualitative studies may be needed to obtain more information on the spectrum of forest exposure as a risk behaviour for malaria in this region.

The Myanmar Ministry of Health offers malaria prevention, diagnosis and treatment to the general population without distinction. This approach is reflected in the high level of bed-net ownership and might contribute to the low levels of transmission observed. However, these data suggest that a strategy tailored to those at highest risk and with asymptomatic infection might be required to eliminate malaria since bed-nets will not protect those working in forest plantations at dawn and dusk [14]. Targeted interventions have been trialed in other areas with varying degrees of success, including: insecticide-treated clothing and hammocks (for use in the forest); toxic mosquito baits; and personal insect-repellents [39]. Targeted mass administration of anti-malarials to specific demographic groups is another option [40]. As yet, none have been evaluated in Myanmar.

This is the first study in Myanmar to combine detailed population-level epidemiological data with highly sensitive molecular techniques. The strength and novelty of this study also lie in the size of this probability sample from an important but unstudied population, with generalizability to Bago Region and central Myanmar. However, higher diagnostic sensitivity has been achieved with the technique of high volume ultra-sensitive qPCR [16] though this requires collection of larger blood volumes by venepuncture, and an unbroken cold-chain for transport of packed red cells to a specialist molecular biology laboratory. Point prevalence of current infection is sensitive to seasonal variation, and this study was conducted towards the end of the rains—regarded as the malaria high season—to sample at the peak of malaria transmission (though different seasonal peaks have been reported for some areas nearby [41, 42]). Moreover, others report little by way of seasonal variation [43], and by correlating PCR findings with serology, which is less sensitive to seasonal changes, robust hypotheses can be developed. The study aimed to minimize bias in this population-based study by weighting the sample so that it was broadly representative of national demographic data in Myanmar in terms of gender and age [44], but the sampling strategy excluded the population living closest to the flooded Sittang River due to adverse weather conditions, which made these areas inaccessible, and where malaria transmission might be higher. In addition, the ethnic composition of this sample differs from national averages because ethnicity in Myanmar is clustered [44]; the sample over-represented Indian participants (33% compared to 2% in national statistics) and underrepresented Shan (3% compared to 9%). One-fifth of residents were absent during study visits, and these individuals were more likely to be male and less likely to be of Indian origin, and this household survey did not include hospital in-patients being treated for malaria. We were only able to provide population weighting at the ward level due to a lack of available population statistics for individual villages. In our experience, village sizes did vary, though it is unlike that this would have affected our prevalence estimates, given the ward-level weighting applied and the small number of infections detected. The overall effect of our diagnostic techniques and sampling strategy is that the study design might have underestimated rather than overestimated malaria prevalence.

Conclusion

Malaria transmission in this area was low. However, there were low numbers of asymptomatic carriers who probably play an important role in maintaining local transmission, and many were infected with *P. knowlesi*. A higher proportion of K13 mutations among *P. falciparum*

cases were observed than has previously documented in this region, and working age men appear at significantly higher risk of malaria exposure when compared to the general population, which needs further research. However, the public health implications are clear; the WHO goal of malaria elimination in this region will require identification and treatment of asymptomatic carriers among high-risk populations in order to control artemisinin resistance and successfully eliminate malaria in this setting.

Abbreviations

PCR: polymerase chain reaction; K13: propeller region of the kelch protein on chromosome 13 of the *Plasmodium falciparum* genome (mutations here may be responsible for conferring artemisinin resistance); RDT: rapid diagnostic test for malaria; WHO: World Health Organization; SCR: seroconversion rate (the rate at which individuals develop antibodies to malaria antigens).

Authors' contributions

IG, TTWH, HMTH, DLH and NF designed the study protocol with advice from TH, TSL and MML. IG, TTWH, HMTH and YN collected the data and coordinated the field teams. IL and CS conducted the PCR analysis and K13 testing. TH and CD performed the ELISA analysis. JC performed the statistical analysis. IG, JC, RG and NF wrote the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Ethical approval

The study received ethical approval from the Research Ethics Committee of University College London (4286/001), the Myanmar Ministry of Health (Letter no. 36) and Department of Medical Research, Lower Myanmar (Letter no. 19). Local and regional health authorities were informed. Informed consent was obtained from all adults and from guardians of children aged under 15. For those aged between 15 and 18, informed consent from both parent and child was obtained. Febrile participants were, with consent, reported to the Malaria Inspector and treated according to national guidelines.

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MAJOR ARTICLE

Contribution of *Plasmodium knowlesi* to Multispecies Human Malaria Infections in North Sumatera, Indonesia

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Background. As Indonesia works toward the goal of malaria elimination, information is lacking on malaria epidemiology from some western provinces. As a basis for studies of antimalarial efficacy, we set out to survey parasite carriage in 3 communities in North Sumatera Province.

Methods. A combination of active and passive detection of infection was carried out among communities in Batubara, Langkat, and South Nias regencies. Finger-prick blood samples from consenting individuals of all ages provided blood films for microscopic examination and blood spots on filter paper. *Plasmodium* species were identified using nested polymerase chain reaction (PCR) of ribosomal RNA genes and a novel assay that amplifies a conserved sequence specific for the *sicavar* gene family of *Plasmodium knowlesi*.

Results. Of 3731 participants, 614 (16.5%) were positive for malaria parasites by microscopy. PCR detected parasite DNA in samples from 1169 individuals (31.3%). In total, 377 participants (11.8%) harbored *P. knowlesi*. Also present were *Plasmodium vivax* (14.3%), *Plasmodium falciparum* (10.5%) and *Plasmodium malariae* (3.4%).

Conclusions. Amplification of *sicavar* is a specific and sensitive test for the presence of *P. knowlesi* DNA in humans. Subpatent and asymptomatic multispecies parasitemia is relatively common in North Sumatera, so PCR-based surveillance is required to support control and elimination activities.

Keywords. Malaria; Indonesia; *Plasmodium knowlesi.*

Malaria remains widespread across Southeast Asia. In Indonesia, 2 million cases of malaria are reported each year, with *Plasmodium falciparum* and *Plasmodium vivax* the 2 major reported causes [1]. Among other species contributing to human infections, *Plasmodium malariae* malaria may require hospitalization in the eastern province of Papua [2] but is not frequently encountered in western Indonesia. *Plasmodium knowlesi,* a parasite of long-tailed and pig-tailed macaques, is also known to infect humans. The morphological features in the blood stage are similar to those seen in *P. falciparum* and *P. malariae*, which in routine practice has led to frequent misdiagnosis [3–5]. High *P. knowlesi* parasitemia occurs in some individuals and has been reported to cause fatal disease [6]. Despite this, a proportion of *P. knowlesi* infections are asymptomatic and submicroscopic across all age groups [7]. A small number of human cases of *P. knowlesi* malaria have been documented in the province of Kalimantan, Indonesian Borneo [8] and in Aceh province [5], but this species has not yet emerged as a major cause of human malaria and is not considered in Indonesian government guidelines.

The Ministry of Health of Indonesia has implemented malaria control, aiming for elimination by 2030. Malaria surveillance relies on passive case detection by microscopic examination and rapid diagnostic tests (RDTs) at primary health care centers [9]. These tests are sufficient to detect clinical malaria infection caused by the 2 major species in Indonesia, *P. falciparum* and *P. vivax* [10]. However, identification of less common species, particularly at low-density parasitemia, is more difficult, which can lead to underdiagnosis [11]. Modeling of data from low-endemicity areas predicts that submicroscopic parasites may contribute 70%–80% of all malaria infections [12], and in vivo studies demonstrate that these contribute to ongoing malaria transmission [13]. Hence, the use of routine microscopy and RDTs in malaria surveillance fails to detect a substantial proportion of the human reservoir of infection and so may compromise malaria elimination strategies. One solution is to deploy molecular assays for parasite detection, because these can provide excellent sensitivity and specificity [14–17].

In preparation for a study of antimalarial drug efficacy in vivo, we performed intensive malaria screening in 3 regencies of the Province of North Sumatera, western Indonesia. In

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addition to microscopy, we used established polymerase chain reaction (PCR) assays [18]. However, these tests have limitations for the identification of *P. knowlesi* infection, because the target region of the 18S ribosomal RNA (rRNA) can cross-react with *P. vivax* [19]. Therefore, we developed a sensitive and highly *P. knowlesi*–specific nested PCR assay to ensure reliable determination of all *Plasmodium* species, including submicroscopic infection, in our study areas.

METHODS AND MATERIALS

Study Sites

A parasitological survey was conducted between January and June 2015 among persons attending outpatient clinics temporarily established in >80 localities across 3 selected regencies in North Sumatera province, Indonesia: Batubara, Langkat, and South Nias regencies (Figure 1). North Sumatera has a total area of 71 680.68 km^2 with a population of 13 215 401. The province is subject to stable low malaria transmission and is currently planning for elimination by 2020. The 3 regencies were selected based on published malaria endemicity data [20]. Batubara, situated on the east coast facing peninsular Malaysia, comprises semiforested and plantation areas. Langkat is a forested highland area (altitude, 105–530 m above sea level), and South Nias is a cluster of islands in the Indian Ocean. Each regency is served by a district hospital and peripheral health clinics, but some rural villages in the study areas have very limited access to these services.

Ethics Approval

The study was approved by the ethics committees of the University of Sumatera Utara, Indonesia (identifier 401/KOMET/FK USU/2014) and the London School of Hygiene & Tropical Medicine, United Kingdom (identifier 8504-01).

Sampling

Sampling strategies differed among the 3 sites, owing to contrasting geography and inconsistencies in access to health facilities. In Batubara, most communities had good access to a health clinic, which was open to patients from 8 am to noon, 6 days per week. We established a 24-hour, 7-day clinic for an 8-week period of screening, after intensive health promotion and education on malaria and sensitization concerning to our study objectives. This sensitization was facilitated by local leaders and carried out at the level of the whole community, but clinic attendance was entirely voluntary. Thus, sampling was not designed to reach the whole community. Local health clinics were also

Figure 1. Map of North Sumatera province, Indonesia. The 3 studied regencies (Batubara, Langkat, and South Nias) are indicated.

asked to refer patients with a malaria diagnosis to our team. Langkat is a forested area, with isolated villages that have poor access to health facilities; we therefore adopted a village-by-village approach, in which a sensitization meeting was followed by 2–3 days of screening, before the team moved to another village. The 31 communities we sampled in South Nias were spread across several islands, and sea journeys were required to move our team and the samples between each village and our study clinic/diagnostic laboratory, which was temporarily set up in a central location on Tello Island.

Malaria testing was done in 3 groups of persons. First, patients with fever (axillary temperature, ≥37.5°C) or a history of fever in the preceding 48 hours who presented at the health clinics were tested for malaria. Tests were also offered to healthy individuals who, after our community sensitization activity, came for a voluntary malaria check. This second group includes children who volunteered during school sensitization visits (Batubara and South Nias only). Third, household members of any individuals who were slide positive for malaria parasites were subsequently visited and also offered malaria testing. Finger-prick blood samples were taken from all participants for thick and thin blood films for microscopy examination (single reading), and blood was also spotted onto filter papers (3MM Whatman) for molecular analysis. Participants confirmed as malaria positive by microscopy were clinically assessed and treated according to national guidelines, regardless of symptoms.

Laboratory Procedures

DNA Extraction

Parasite DNA was extracted from filter papers using the Chelex method [21, 22].

Parasite Species Identification by rRNA Gene Amplification

A conventional nested PCR assay targeting the genes encoding the *Plasmodium* 18S rRNA was performed on all samples for species determination and detection of submicroscopic infections [3, 18]. Positive controls for *P. falciparum*, *P. vivax*, *P. malariae*, *Plasmodium ovale* spp. and *P. knowlesi* were included in all nested PCR assays.

Development and Validation of a Novel Highly Specific P. knowlesi *PCR Assay*

To overcome the cross-reactivity between *P. knowlesi* and *P. vivax* that occurs in the rRNA gene PCR assays, we developed a hemi-nested PCR assay based on a conserved region of the amino-terminal exon of the 50–70 members of the gene family encoding *P. knowlesi*–specific schizont-infected cell agglutination variant antigens (SICAvar) [23]. The following primers were used: first amplification, SICAf1, 5'-GGTCCTCTTGGTAAAGGAGG-3'and SICAr1, 5'- CCCTTTTTGACATTCGTCC-3'; second amplification, SICAf2, 5'-CTTGGTAAAGGAGGACCACG-3' and SICAr1; these generated a final amplicon of 228–249 base pairs, encoding 76–83 amino acids. This sequence occurs in both types I and type II *sicavar* genes [23, supplementary table 1]. For the first round of amplification, 5 μL of DNA template was used in a total volume of 25 μL, and 0.2 μL of this product was the template for the 25-μL reaction mixture of the hemi-nested amplification round. Amplifications were performed under the following conditions: 94°C for 3 minutes, then 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 65°C for 1 minute, with extension at 65 °C for 5 minutes.

This assay was tested against control DNA from all human malaria parasites (*P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale curtisi,* and *P. ovale wallikeri*), simian malaria parasites (*P. knowlesi* and *Plasmodium inui*, *cynomolgi*, *coatneyi,* and *fieldi*), clinical *P. knowlesi* isolates obtained from Kapit of Malaysian Borneo, and human DNA from malaria-free individuals to assess specificity. To determine the limit of detection of the assay, *P. knowlesi* culture of known parasitemia (kindly provided by F. Mohring) was serially diluted in whole human blood and spotted on filter paper, and DNA was extracted with a QIASymphony automated DNA extraction system. All field isolates were tested for *P. knowlesi* infection using this novel PCR assay. The *sicavar* amplicons from a subset of samples were verified by direct sequencing using BigDye Terminator v3.1 cycle sequencing kits and analysis on an ABI 3730 Sequencer (Applied Biosystems). Results were aligned and compared with the *P. knowlesi* strain H reference genome, using Geneious (version 8.0.5) and BLAST (Basic Local Alignment Search Tool) software.

RESULTS

Validation of SICAvar Assay for *P. knowlesi*–Specific Identification

The novel SICAvar gene assay was validated against DNA from a range of *Plasmodium* species DNA and from in vitro cultured *P. knowlesi*. The test was found to be specific, because the primers did not generate bands from the DNA of any of the *Plasmodium* species tested other than *P. knowlesi* (Figure 2). The *sicavar*-targeted primers detected 377 *P. knowlesi* infections, suggesting significantly higher sensitivity than the rRNA assay, which identified only 76 *P. knowlesi*–infected individuals (Table 1). Furthermore, comparison of the results showed that only 10 persons (2.3%) had a positive test result in both assays. The finding that 66 individuals were *P. knowlesi* positive by rRNA nested PCR alone suggests that the test cross-reacts with *P. vivax* DNA in the absence of *P. knowlesi* under the conditions used in this study.

Because we did not deploy a "tie-breaker" test for *P. vivax*, we cannot say for certain whether the *P. vivax* rRNA gene primers also cross-react with *P. knowlesi* DNA. We did find that 18 individuals were positive for both *P. vivax* and *P. knowlesi* rRNA amplicons in the nested PCR. Given this ambiguity, and the fact we were not able to repeat test the sample set, we proceeded with analysis taking the *sicavar* assay result as definitive for

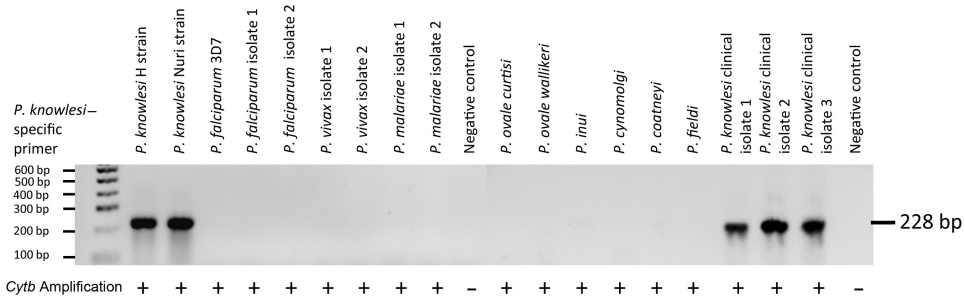

Figure 2. Validation of *Plasmodium knowlesi* primers targeting *sicavar* against human and simian malaria parasites reported from Southeast Asia. Control DNA of human malaria *Plasmodium* species were from imported cases in the United Kingdom (courtesy of the Public Health England Malaria Reference Laboratory); 2 isolates each are shown for *Plasmodium falciparum, Plasmodium vivax,* and *Plasmodium malariae.* Genus-specific primers for the cytochrome B gene (*cytb*) were used to confirm presence of detectable *Plasmodium* DNA in each sample, indicated by plus signs; bp, base pairs.

P. knowlesi. Nearly half of all *sicavar*-positive *P. knowlesi* infections were also rRNA amplicon positive for ≥1 other species, *P. vivax* being the most common coinfection (Table 1). The limit of detection of the assay, as performed on dried filter paper blood samples, was estimated as 0.1 parasite per µL of whole blood (data not shown).

As further confirmation of our results, 7 *P. knowlesi* isolates detected by SICAvar were PCR amplified, and the products directly sequenced. The sequences exhibited high variability, as expected for variant antigens, even in this most conserved exon (Figure 3). Interestingly, 2 of the 7 sequences obtained harbored an insert encoding an additional 7 amino acids. These 2 forms were used to probe the current *P. knowlesi* reference genome (http://www.sanger.ac.uk/resources/downloads/protozoa/plasmodium-knowlesi.html). and both queries identified a number of distinct sequences in the reference genome (Supplementary Table 1).

After this successful validation of the SICAvar PCR assay, we were able to deduce robust estimates of the contribution of each species to malaria infections in each of our 3 sites. The most abundant in both Langkat and South Nias were *P. falciparum* and/or *P. vivax*, as expected. However in Batubara, *P. knowlesi* (39.7%) was more abundant than *P. vivax* (35.1%) among our tested participants (Figure 4). Of patients reporting fever symptoms in the previous 72 hours, 22 were carrying *P. knowlesi* monoinfection by PCR, with another 47 having *P. knowlesi* double infections with *P. vivax* (n = 20), *P. falciparum* (n = 19), or *P. malariae* (n = 2) or various combinations of triple-species infection.

Table 1. Comparison of 2 PCR Assays for Plasmodium knowlesi Case Detection

	Cases, No. (%)			
Infection Detected	18S rRNA Assay	SICAvar Assay	Any Assay	Both Assays
Total *P. knowlesi* cases	76 (100)	377 (100)	443 (100)	10 (100)
P. knowlesi monoinfection	42 (55.3)	215 (57.0)	254 (57.34)	3 (30)
P. knowlesi plus *P. vivax*	16 (21.1)	65 (17.2)	77 (17.38)	4 (40)
P. knowlesi plus other *Plasmodium* spp. infections	18 (23.7)	97 (25.7)	112 (25.28)	3 (30)

Abbreviations: PCR, polymerase chain reaction; rRNA, ribosomal RNA; SICAvar, schizont-infected cell agglutination variant antigen.

Parasite Carriage

A total of 3731 individuals from Batubara (n = 1270), Langkat (n = 544), and South Nias (n = 1917) were included in the malaria screening (Supplementary Table 2). In these 3 regencies, 117 (9.2%), 98 (18.0%), and 397 (21.3%) individuals, respectively, were positive for *Plasmodium* infections by microscopy. Three species (*P. falciparum*, *P. vivax,* and *P. malariae)* were identified. A considerable number of participants with malaria-positive slides were negative by PCR subsequently performed on stored blood spots. Thus, the total number of patent infections confirmed by both microscopy and PCR decreased to 93 (8.1%), 74 (13.6%) and 169 (9.1%), respectively, indicating poor specificity of microscopy in South Nias in particular. Conversely, a substantial number of submicroscopic infections were identified by PCR alone, with a PCR-confirmed parasite carriage in 25.2%, 33.5%, and 34.8% of tested individuals, respectively, in the 3 sites (Table 2). All *Plasmodium* species with the exception of *P. ovale* spp. were detected among our samples by a combination of the rRNA gene and SICAvar PCR assays.

Carriage of Submicroscopic Infections

PCR analysis revealed that the majority of the 1169 infected individuals (71.3%) harbored submicroscopic parasites (Table 2). Among these submicroscopic infections, 77.7% (647 of 833) were single-species infections predominated by *P. vivax* (n = 227) and *P. knowlesi* (n = 220). Submicroscopic infections of any species were more often found in older individuals, the mean age in this group being 23.0 years, (95% confidence interval [CI], 21.7–24.3 years). The mean age of individuals with patent infections was significantly lower, at 18.0 years

Figure 3. Sequence alignment of representative 120 and 141 nucleotide sequences of *sicavar* amplicons from the peripheral blood DNA of 4 participants from Batubara (BB) and 2 each from Langkat (LK) and South Nias (NS). Amplicons were produced by heminested polymerase chain reaction, as described in Materials and Methods. Sample order is determined by the alignment. Representative amplification products were chosen for this sample and sequenced directly using amplification primers to prime forward and reverse sequencing reactions. Sequences shown were confirmed in both directions. Two loci from the *Plasmodium knowlesi* strain H reference genome (PKNH_0932000, type I schizont-infected cell agglutination variant antigen (SICAvar), chromosome 9, centrally located; PKNH_0118500, type II SICAvar, chromosome 1, subtelomeric) are shown for comparison. Double peaking was seen in some samples; only the peak with highest amplitude was read for this analysis.

(95% CI, 16.2–19.7 years; $P < .001$, 2-sided t-test) (Table 3). Submicroscopic carriage was observed more often in Batubara (odds ratio, 1.67; 95% CI, 1.14–2.45) or South Nias (2.02; 1.43–2.85) than in Langkat. However, individuals with multispecies infections were not significantly older than those infected with a single species ($P = .66$, 2-sided *t* test).

DISCUSSION

In this study, a total of 3731 febrile and nonfebrile residents of 3 regencies in North Sumatera province were screened for malaria infection by both microscopy and PCR detection of parasite DNA. Microscopy identified 612 infected participants, whereas PCR identified 1169 individuals harboring ≥1 of the 4 *Plasmodium* species identified: *P. falciparum*, *P. knowlesi*, *P. malariae,* and *P. vivax*. Using a novel assay developed for this study, which detects a conserved motif in the multicopy *sicavar* gene family, we found that *P. knowlesi* was present in 377 individuals (10.1%), including both patent and subpatent infections. *P. vivax* and *P. falciparum* were both frequently detected, occurring in 11.3% and 8.2% of all individuals tested.

Although *P. knowlesi* infection has been widely recorded in Southeast Asia, only a handful of confirmed cases have been from Indonesia, from Kalimantan, eastern Borneo [8, 24], and, more recently, 20 cases from Aceh province, on the northwest extremity of Sumatera [5]. This latter study of 1532 individuals used a combination of passive and reactive case detection to identify a total of 20 *P. knowlesi*, 15 *P. vivax,* and 8 *P. falciparum* infections, almost all of which were symptomatic. This contrasts with our findings of more frequent parasite carriage, and a significant proportion of subpatent and asymptomatic infections.

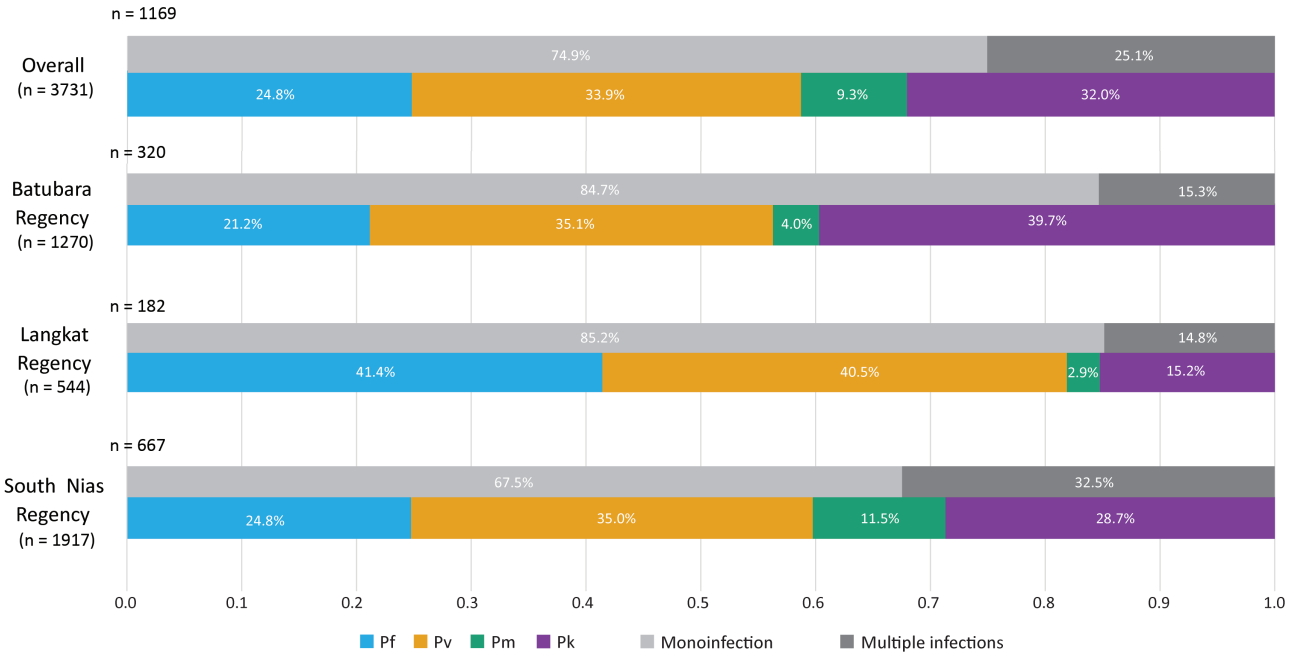

Figure 4. Proportion of *Plasmodium* species and multiplicity of infections by regency. The denominator for each site (total number of individuals tested) is given under the regency name, and the number of parasite-positive individuals is shown at the top-left of each bar graph. The horizontal axis represents the proportion of the total number of infections in each bar. Colored bars denote species; gray bars denote proportions of mixed-species infections identified in each site. Abbreviations: Pf , *Plasmodium falciparum*; Pk, *Plasmodium knowlesi*; Pm, *Plasmodium malariae*; Pv, *Plasmodium vivax.*

Table 2. Submicroscopic Infections in 1169 Participants With Positive PCR Results for Plasmodium spp.

Infection Type	Participants, No. (%)[a]	
	All PCR Positive	PCR Positive and Microscopy Negative
All *Plasmodium* infections	1169 (31.33)	833 (71.26)
Plasmodium falciparum	247 (6.62)	165 (14.11)
Plasmodium vivax	335 (8.97)	227 (19.41)
Plasmodium malariae	40 (1.07)	35 (2.99)
Plasmodium knowlesi	254 (6.80)	220 (18.81)
Mixed infections	293 (7.85)	186 (15.91)
No. of species by PCR		
1	876 (74.94)	647 (77.67)
2	256 (21.90)	163 (19.57)
3	35 (2.99)	21 (2.52)
4	2 (0.17)	2 (0.24)
PCR positive by regency		
Batubara	320/1270 (25.19)	227/320 (70.93)
Langkat	182/544 (33.45)	108/182 (59.34)
South Nias	667/1917 (34.79)	498/667 (74.66)

Abbreviation: PCR, polymerase chain reaction.

[a]Frequencies are shown together with relative frequency expressed as a percentage of all participants (N = 3731), all PCR-positive participants (n = 1169), or all participants with submicroscopic infections (n = 833). Top-row percentages read horizontally; percentages for other indented categories read vertically within the appropriate subgroup, apart from the regency-specific data (with denominators as shown).

Table 3. Age and Sex of Participants With Positive PCR Results for Plasmodium spp.

Age and Sex by *Plasmodium* Species[a]	PCR Positive, No. (%)
Parasite carriage by age group, y	
P. falciparum	
<5	24 (9.72)
5–14	91 (36.84)
>15	132 (53.44)
P. vivax	
<5	41 (12.24)
5–14	143 (42.69)
>15	151 (45.07)
P. malariae	
<5	4 (10.00)
5–14	18 (45.00)
>15	18 (45.00)
P. knowlesi	
<5	28 (11.02)
5–14	96 (37.80)
>15	130 (51.18)
Parasite carriage among female participants	608 (52.01)
P. falciparum	137 (55.47)
P. vivax	171 (51.04)
P. malariae	15 (37.50)
P. knowlesi	129 (50.79)

Abbreviation: PCR, polymerase chain reaction.

[a]Age and sex data are presented for single-species infection only.

Malaria transmission intensity is much lower in Aceh than in North Sumatera, and this neighboring province is closer to eliminating the disease. North Sumatera's natural forests have been affected by deforestation in the last few years. Residents of rural districts may live in close proximity to semiforested, forested, or plantation areas, with a high likelihood of forest exposure, but may not have adequate access to health facilities and antimalarial medication. In the Aceh study, the more frequent occurrence of symptoms in persons with knowlesi malaria reflects observations in Malaysian Borneo where, as in Aceh, other *Plasmodium* species have decreased in prevalence over the past decade [4, 25]. In North Sumatera, as our data show, *P. falciparum* and *P. vivax* remain common and it may be that acquired immunity to these human parasites protects individuals subsequently infected with *P. knowlesi*, although asymptomatic infections have also been reported in Sabah [7].

Our novel PCR assay identified an unexpectedly large number of *P. knowlesi* infections, and so we made some effort to validate its sensitivity and specificity (Figure 2). SICAvar genes encode an antigen family unique to *P. knowlesi*, estimated to number >100 members, including both multiexon and truncated forms randomly distributed across all 14 chromosomes [23]. SICAvar proteins undergo antigenic variation in the course of a single infection [26, 27] and are likely to play a key role in maintaining chronic parasitemia in semi-immune hosts. Sequencing of a handful of *sicavar* amplicons from our samples confirmed nucleotide diversity in our short target sequence, double peaking indicative of multiple loci being amplified in some cases, and distinguished a variant form with a 7–amino acid insert (Figure 3). Probing the *P. knowlesi* reference genome with these 2 forms generated many hits with both length variants, including both types 1 and 2 loci (Supplementary Table 1). These findings suggest our assay is performing as hoped and is a useful tool for identifying *P. knowlesi* infections among complex mixtures of *Plasmodium* species.

The national malaria control program focuses on case management through passive surveillance at primary health centers, deploying microscopy or RDTs to detect malaria cases to be treated [9]. In our study, microscopy identified many *Plasmodium* spp. infections, but lacked accuracy in distinguishing among the 4 species present, as previously reported in Malaysia [28]. Although personnel differed between sites, identification of the less common species was problematic in all sites. *P. malariae* was only detected in 1 case and *P. knowlesi* was left unrecognized. In South Nias, microscopy results showed poor specificity (Supplementary Table 2), leading to a number of false-positives, whereas specificity was good at the other 2 sites. RDTs have limitations for detection of *P. knowlesi* because they include only *P. falciparum* and *P. vivax* parasite lactate dehydrogenase monoclonal antibodies [11]. Most importantly, almost half of the infections detected with PCR were not identified by

either of the conventional tests. Therefore, although RDTs and microscopy remain satisfactory for diagnosis of symptomatic falciparum and vivax malaria requiring treatment, these are not adequate tools for malaria elimination and control activities, because submicroscopic *Plasmodium* carriage is associated with subsequent transmission to mosquitoes [13].

On the other hand, molecular tests are highly sensitive and specific, provide the capacity to detect low-density infections missed by microscopy or RDTs, and are well established for detection of human malaria infections [11, 29]. To overcome cross-amplification of *P. vivax* isolates with ribosomal gene PCR assays [3, 19], Ghinai et al [30] have recently described a PCR-sequencing approach to detect *P. knowlesi cytb* DNA, which also provided satisfactory sensitivity. However, our *sicavar* target assay provides greater sensitivity for *P. knowlesi* identification in our hands and was effective in detecting submicroscopic parasites. This is a species-specific test, because the *sicavar* gene family is unique to *P. knowlesi* (Figure 2). Because of this improved specificity and sensitivity, we can confidently report moderate numbers of *P. knowlesi* coinfections with *P. vivax* in the present study.

Despite the aim to achieve malaria elimination by 2020 in Sumatera, our study demonstrated that a substantial number of individuals in our study areas carried parasites. *P. falciparum* contributed one-fifth of infections, and *P. vivax* was seen slightly more often. Interestingly, our findings also demonstrate that *P. knowlesi* carriage is not uncommon. Many *P. knowlesi*–infected individuals harbored additional *Plasmodium* species (Table 1), in contrast to areas in Malaysian Borneo where *P. vivax* and *P. falciparum* are now very scarce [4, 7]. Multiple-species infections in our study were equally distributed across all age groups with both female and male subjects exposed to a similar risk of infection (data not shown), whereas submicroscopic infections were more common in older individuals (Table 2), suggesting a role for acquired immunity [31]. The observation of asymptomatic *P. knowlesi* infections in our study is consistent with recent findings in Malaysia [7] but does not necessarily support the occurrence of human-mosquito transmission of *P. knowlesi.*

Macaques were present in all sites, and the communities shared established risk factors for malaria transmission by forest-dwelling *Anopheles,* thought to be the vector of *P. knowlesi*. These data may suggest that acquired immunity permits sustainable chronic infections with this simian parasite. Cross-protection among the 4 human *Plasmodium* species may maintain overall parasite density at low levels [31], which could also plausibly apply to *P. knowlesi,* particularly in settings where the closely related *P. vivax* is present. One weakness of our study is the lack of a systematic sampling procedure, which may have introduced bias; as we had not previously worked in these 3 regions, methods were adapted for each site to reflect the facilities available and particular local challenges. For South Nias, these included frequent sea journeys in small boats. Future studies could deploy a more systematic approach and collect sufficient data to better explain and characterize asymptomatic infections, now that community contacts have been established and baseline information is available.

Our study demonstrated the importance of submicroscopic infections of 4 *Plasmodium* species, including *P. knowlesi*, to malaria transmission in North Sumatera. There is an urgent need for the national malaria program to include in malaria guidelines the recommendation that microscopists are trained to identify *P. knowlesi* infection in Indonesian clinics. Molecular detection of infection is also needed, to strengthen control and elimination programs by accurately defining the true extent of the malaria reservoir, so as to achieve the current goal of elimination [32].

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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